Thuricin CD: a potential therapeutic targeted against *Clostridium difficile*-associated diarrhea (CDAD).

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I hereby declare that this thesis is my own work and has not been submitted for another degree, either at University College Cork or elsewhere.

Signed _____

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LIST OF ABBREVIATIONS

AAD, antibiotic-associated diarrhea

ABC, ATP-binding cassette

ADP, adenosine diphosphate

AFLP, amplified fragment length polymorphism

ATP, adenosine triphosphate

BGSC, *Bacillus* Genetic Stock Centre

BHI, Brain Heart Infusion

BHV, toxin B from hypervirulent strains

BLAST, Basic Local Alignment Search Tool

CDAD, *Clostridium difficile*-associated diarrhea

CDI, *Clostridium difficile* infection

CDT, binary toxin

CdtLoc, binary toxin locus

CFS, cell-free supernatant

CFU/ml, colony forming units per millilitre

CROP, combined repetitive oligopeptide

C-terminal, carboxy terminal

DMSO, dimethyl sulphoxide

DNA, deoxyribonucleic acid

DPC, Dairy Products Research Centre collection

ECDC, European Centre for Disease Prevention and Cure

EDTA, ethylene diamine tetracetic acid

EF, elongation factor

ELISA, enzyme-linked immunosorbent assay

ESCMID, European Society of Clinical Microbiology and Infectious Diseases

FAA, Fastidious anaerobic agar

FDA, Food and Drug Administration

FIC, fractional inhibitory concentration

FMT, faecal microbiota transplantation

GIT, gastrointestinal tract

GRAS, generally regarded as safe

GTP, guanosine triphosphate

HD, human defensin

HIV, human immunodeficiency virus

HNP, human neutrophil peptide

IBD, inflammatory bowel disease

IDSA, Infectious Diseases Society of America

IgA, immunoglobulin A

IgG, immunoglobulin G

IPTG, isopropyl β-D-thiogalactopyranoside

IVIG, intravenous immunoglobulin

K22T, lysine to threonine substitution at residue 22

LB, Luria Bertani

LCT, large clostridial toxins

M21V, methionine to valine substitution at residue 21

Man-PTS, mannose phosphotransferase system

MIC, minimum inhibitory concentration

MLS, macrolide lincosamide streptogramin

MLST, multi-locus sequence typing

MLVA, multi-locus variable number of tandem repeat analysis

NCBI, National Centre for Biotechnology Information

NGS, next generation sequencing

NSR, nisin resistance protein

N-terminal, amino terminal

NVB, Novacta Biosystems Ltd

OD, optical density

ORF, open reading frame

PaLoc, pathogenicity locus

PBP, penicillin binding protein

PCR, polymerase chain reaction

PFGE, pulsed-field gel electrophoresis

PMC, pseudomembranous colitis

PPI, proton pump inhibitor

R027, ribotype 027

RBS, ribosome binding site/sequence

RCM, Reinforced Clostridium Medium

REA, restriction endonuclease analysis

RFLP, restriction fragment length polymorphism

RNA, ribonucleic acid

RP-HPLC, reverse phase-high performance liquid chromatography

rRNA, ribosomal ribonucleic acid

SAM, S-adenosylmethionine

SHEA, Society for Healthcare Epidemiology of America

SNP, single nucleotide polymorphism

TcdA, toxin A

TcdB, toxin B

TFA, trifluoroacetic acid

TMD, transmembrane domains

tRNA, transfer ribonucleic acid

V15F, valine to phenylalanine substitution at residue 15

Abstract

Clostridium difficile has caused significant morbidity and mortality over the last two decades, especially in North America and Europe. The perturbations caused to the gut microbiota due to broad spectrum antibiotics and consequent lack of protection provided by the commensal microbes is considered the main risk factor for *C. difficile*-associated diarrhea (CDAD). The emergence of hypervirulent *C. difficile* strains has exacerbated the problem over the last decade while the continuing problem of recurrence of CDAD following metronidazole/vancomycin administration has led to investigations into alternative/adjunctive therapeutic options for the disease. The relatively recent advancements in next generation sequencing (NGS) technology have aided scientists to understand and appreciate the extensive damage inflicted on the gut microbiota by broad spectrum antibiotics.

As mentioned, due to recurrence of CDAD, as well as the possibility of metronidazole/vancomycin resistance amongst *C. difficile*, alternative antimicrobials and antimicrobial combination therapies warrant investigation. Bacteriocins are ribosomally synthesised peptides produced by bacteria, which can have either narrow or broad spectrum of activity against other bacteria. Thuricin CD is one such bacteriocin, which has an extremely narrow host range, restricted mainly to *C. difficile* and some *Listeria monocytogenes* and *Bacillus* strains. Thuricin CD belongs to a novel class of bacteriocins referred to as sactibiotics. Thuricin CD displays potent activity against all *C. difficile* isolates tested to date, while having minimal impact on members of the gut microbiota. Since the main etiological agent of CDAD and recurrence is dysbiosis caused by broad spectrum antibiotics, the narrow spectrum thuricin CD has the potential to replace conventional broad spectrum antibiotics. Antimicrobial combinatorial therapy has also attracted some attention in

recent years as a means of treating infections. It is plausible that resistance amongst target strains is less likely to develop when a combination of antimicrobials with different modes of action is used. Indeed, a similar concept of hurdle effects has existed in the food industry for years, whereby two or more antimicrobials/stressors are combined to inhibit the growth of pathogenic/spoilage bacteria. Here, following an initial determination of minimum inhibitory concentrations of five antimicrobials against nineteen *C. difficile* clinical isolates, it was observed that the sactibiotic thuricin CD when combined with the antibiotic ramoplanin, as well as the lantibiotic actagardine when combined with ramoplanin, function in a partially synergistic/additive manner against the majority of *C. difficile* targets. Other partial synergistic effects were also apparent in this study. The advantage of such antimicrobial combination therapies with bacteriocins include: i) reducing the concentration of antibiotics required, thus decreasing the chances of side effects, ii) attenuating the likelihood of resistance development amongst target strains due to two different modes of action of the two antimicrobials combined, iii) reducing the financial burden associated with the administration of more expensive antibiotics. Following on from such initial antimicrobial combination studies, the development of resistance to the bacteriocin thuricin CD was investigated in depth with the ultimate view to gain better insights into the mode of action of the bacteriocin. Different ribotypes of *C. difficile* strains, as well as thuricin CD-sensitive *L. monocytogenes* and *Bacillus firmus* strains were employed to this end. It emerged that low-level resistance development to thuricin CD was possible through incremental exposure to increasing concentrations of thuricin CD in a stepwise manner. Phenotypic analysis of such mutants revealed minor alterations in sensitivities of some of the thuricin CD-resistant mutants to the β-lactam group of

antibiotics compared to the wild type parental strains, suggesting that altered expression levels of penicillin binding proteins may be involved in resistance development. Furthermore, altered growth rates of *L. monocytogenes* resistant mutants on the sugars mannose and glucose suggested that the mannose phosphotransferase system could be a possible receptor for thuricin CD.

Bacteriocin producers contain self-defense mechanisms to protect themselves from the antimicrobial activity of the bacteriocin they produce. Such innate immunity systems generally contain either an ABC transporter system, a dedicated immunity protein or a combination of both systems. Here, following detailed *in silico* analysis of the thuricin CD gene cluster and downstream experimental analysis, a novel immunity protein designated TrnI was identified. The experimental findings were consistent with *in silico* investigations, establishing that TrnI is a small hydrophobic immunity protein with two transmembrane domains. It was apparent that TrnI plays a key role in immunity to thuricin CD, both independently and in combination with an ABC transporter system. Truncated versions of TrnI highlighted that the N-terminus of the protein contains the most important domains for functionality. Finally, the entire thuricin CD gene cluster (containing eight genes involved in thuricin CD production, post-translational modification, export and immunity) was expressed heterologously in *Bacillus subtilis*. Such heterologous production of thuricin CD not only experimentally established which genes are responsible for thuricin CD production, it also led to the development of a heterologous host system in which the functions of the individual genes could be ascertained.

Due to the studies presented in this thesis, it is highly likely that strategies will emerge that will eventually facilitate the generation of enhanced derivatives of thuricin CD and will help to gain an insight into the specific residues of importance

within Trnα and Trnβ. It is possible that any heterologous host expressing thuricin CD may become overwhelmed by the presence of the bacteriocin, affecting the viability of the host. The identification of the novel immunity gene *trnI* may lead to the development of heterologous host systems with a hyper-immune state through introduction of additional copies of the immunity gene, and could result in the overexpression of thuricin CD, thereby leading to increased yield. Bioengineering of the immunity protein TrnI via site-directed/random mutagenesis could also lead to the identification of a derivative of TrnI with enhanced immunity which could have applications for the over-production of thuricin CD. The discovery of effective antimicrobial combinations and encouragingly, lack of any antagonistic combinations against *C. difficile* presented in this thesis may eventually prove to be alternative therapeutic options for CDAD in the clinic. A thorough understanding of the precise mechanisms of thuricin CD resistance development and investigations into its mode of action may reveal a specific receptor that thuricin CD binds to. Elucidation of the mode of action of thuricin CD will help to explain the narrow spectrum of activity of thuricin CD against *C. difficile*. This may help us gain insights into the structure-function and ligand-receptor binding characteristics of the thuricin CD peptides and eventually lead to the development of bioengineered derivatives of bacteriocins or 'designer antimicrobials' tailored specifically towards *C. difficile.*

CHAPTER I

Clostridium difficile-associated infection, pathogenesis and therapeutic options.

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Abstract

Clostridium difficile is mainly a nosocomial pathogen and is a significant cause of antibiotic-associated diarrhoea. It is also implicated in the majority of cases of pseudomembranous colitis. Recently, advancements in next generation sequencing technology (NGS) have highlighted the extent of damage to the gut microbiota caused by broad-spectrum antibiotics, often resulting in *C. difficile* infection. Furthermore, over the last decade, the emergence and spread of 'hypervirulent' *C. difficile* isolates producing higher amounts of toxins as well as binary toxin, such as strain R027, has been a cause for concern. The increased severity of disease caused by such epidemic-associated strains compounded by their increased resistance to fluoroquinolone, exacerbate the problem. Currently the treatment of choice for *C. difficile*-associated diarrhoea (CDAD) involves the use of metronidazole and vancomycin. However, recurrence and relapse of CDAD, even after rounds of metronidazole/vancomycin administration is a problem that must be addressed. The efficacy of alternative antibiotics such as ramoplanin, fidaxomicin, rifaximin and nitazoxanide, as well as faecal bacteriotherapy has been assessed and some have yielded positive outcomes against *C. difficile*. Some bacteriocins have also shown promising effects against *C. difficile* in recent years. In light of this, the mechanisms of colonization, pathogenesis and recent advancements in therapeutic options against CDAD are discussed in this review.

Introduction

Clostridium difficile is a Gram-positive anaerobic sporeformer and is the etiological agent responsible for *C. difficile*-associated diarrhoea (CDAD). *C. difficile* was initially considered a harmless commensal of the gastrointestinal tract of infants, when originally isolated in 1935 but its role in nosocomial diarrhoea and pseudomembranous colitis (PMC) was appreciated only in the 1970s (1, 2). The development of antibiotics for the treatment of infectious diseases in the 20th century has been a significant accomplishment. However, it is ironic that antibiotics, and in particular broad spectrum antibiotics, are the main etiological agents of one of the most notorious nosocomial infections, CDAD. CDAD has significant financial implications, and an estimated €5000-15000 is spent per CDAD case in England and approximately €3 billion per year in the EU in total. The corresponding figure is about €2-4 billion per year in the US (3, 4). The majority of *C. difficile* strains produce toxin A and toxin B, which are responsible for the clinical manifestation of the disease. The recent emergence and widespread dissemination of 'hypervirulent' outbreak-associated *C. difficile* strains have caused problems for clinical practitioners. Furthermore, the ongoing problem of CDAD recurrence post-antibiotic therapy, caused by perturbations of the gut microbiota, has encouraged scientists to seek alternative therapeutic options. Perhaps the most potent defence against CDAD is the maintenance/restoration of a fully intact gut microbiota, providing protection through a complex process referred to as 'colonization resistance.'

This review focuses on the processes involved in *C. difficile* pathogenesis, its virulence factors and the emergence of toxin-variant strains and outbreak-associated strains. Traditional approaches and the recent developments in novel *C. difficile* therapeutic options are also discussed. In particular, we focus on antibiotics and

adjunctive therapeutic options which have the potential to replace the current standard metronidazole and/or vancomycin therapy. In this regard, recent *in vivo* studies and clinical trials conducted with alternative and/or adjunct anti-*C. difficile* therapeutic options are discussed.

COLONIZATION

Colonization resistance

The use of antibiotics acts to perturb the host-microbiota homeostasis and can lead to CDAD, particularly in immunosuppressed patients (5). In the late 1970s and early 1980s, it was hypothesised that antibiotics contributed to the development of CDAD by means of disruption of a complex series of events referred to as 'colonization resistance (6, 7).' Indeed, the concept of 'colonization resistance' was first recognised as early as the 1940s when it was found that the administration of streptomycin led to a marked change in the cultivable bacteria which were recovered from the faeces of mice (8). An association between antibiotic-mediated disruption of the gut microbiota and onset of CDAD was noted when Bartlett et al. identified *C. difficile* to be the causative agent of clindamycin-associated colitis (5). Indeed, this overgrowth of *C. difficile* following antibiotics had also been shown in hamsters and mice (5, 7). Studies also demonstrated that the commensal gut microbiota of hamsters was able to inhibit *C. difficile* in germ-free mice and continuous flow cultures (7, 9).

C. difficile is implicated in approximately 20-30% of cases of antibiotic-associated diarrhoea (AAD) and in approximately 90% of cases of PMC (10). However, it is implicated in approximately 0.9% of hospital infections overall according to the US Agency for Healthcare Research and Quality. Antibiotics which predispose to CDAD include clindamycin, the cephalosporins and the aminopenicillins (11, 12). Unsurprisingly, it has also been found that consistent exposure to antibiotics regularly over a period of time leads to reduced 'colonization resistance' to *C. difficile*, most likely attributed to the extensive damage caused by broad spectrum antibiotics (13, 14). As noted above, recurrence of CDAD has been a serious problem. Recurrent CDAD can be defined as diarrhoea that is associated with a positive *C. difficile* test and occurs days to weeks after completion of treatment of an initial CDAD episode (15). Patients with recurrent CDAD have a markedly altered indigenous microbiota, which is postulated to inhibit the restoration of 'colonization resistance' and thus, increases the risk of developing CDAD again (13). In support of this, recent findings have suggested that faecal transplantation may be effective as a means of preventing recurrent CDAD (16). However, it is still not a Food and Drug Administration (FDA)-approved therapy and an inherent risk of transmission of pathogens is associated with a procedure of this nature. A rise in the number of cases of recurrent CDAD has prompted researchers to try to elucidate the mechanisms by which *C. difficile* is suppressed by the host commensal microbiota and to harness this knowledge to develop new therapies.

Mechanisms of colonization resistance and CDAD

The gut microbiota contributes in the transformation of bile acids in the small intestine. This transformation of bile acids has significant effects on the germination of *C. difficile* spores and their subsequent outgrowth. Bile salt hydrolase enzymes act to deconjugate bile acids from their amino acid moieties (17). These enzymes may also act to inactivate the antibacterial actions of bile. Bacteria also transform primary bile acids to secondary bile acids using the enzyme 7-dehydroxylase (17). Therefore, the commensal gut microbiota has an important role in altering the composition of bile in the intestine and may affect the antimicrobial properties of bile. *In vitro* studies have demonstrated that bile stimulates *C. difficile* spore germination (18). Indeed, taurocholate and glycine have been shown to promote the germination of spores by a thousand-fold, whereas the secondary bile acid deoxycholate is toxic to vegetative *C. difficile* cells (19). The use of broad spectrum antibiotics causes a decrease in the levels of deoxycholate, and an increase in cholate levels, and stimulates the germination of *C. difficile* spores by reducing bacteria which generate deoxycholate (19).

Risk factors for CDAD

The use of broad spectrum antibiotics is a key contributory factor to developing CDAD. Such broad spectrum antibiotics are associated with dysbiosis, or perturbations in the human gut microbiota (20). Indeed, over the last number of years, the development of next-generation sequencing (NGS) has aided in comprehending the extent of this damage caused by broad spectrum antibiotics. Several studies have reported the effects of broad spectrum antibiotics on the gut

microbiota, employing the use of clone libraries and downstream Sanger sequencing (21-23).

With regards to *C. difficile*, other than inflicting damage to the gut microbiota, antibiotics such as (desacetyl)cefotaxime and clindamycin stimulate the sustained germination of *C. difficile* spores and consequent production of toxins (24, 25). While cefoxtaxime and desacetylcefotaxime induce germination of *C. difficile* during administration, clindamycin has been found to stimulate *C. difficile* germination and toxin production 8 days after the antibiotic is discontinued (24, 25). Therefore, it would appear that germination of *C. difficile* is promoted when clindamycin is present at concentrations below the minimum inhibitory concentration (MIC) (24, 26). In addition to clindamycin and cephalosporins, other antibiotics that predispose to CDAD include fluoroquinolones, aminopenicillins, vancomycin and metronidazole (27). Even the antiviral valacyclovir has been associated with a case of *C. difficile* colitis (28). The use of proton pump inhibitors (PPIs) in particular, as well as histamine H2-receptor antagonists has also been suggested to lead to an elevated risk of acquiring CDAD. However, it is not yet abundantly clear whether the apparent increased risk of CDAD due to such gastric acid production inhibitors is a direct or indirect effect or whether other underlying causes are involved as well (29). One report indicated that patients being treated with PPIs are approximately four times more likely to suffer from recurrent CDAD than patients not in receipt of PPIs (30). There are a number of other cohorts of patients that seem to be at an increased risk of developing CDAD. The number of reported cases of CDAD in peri-partum women is rising, while post-transplantation patients seem to be more susceptible to CDAD as well (31). Patients suffering from inflammatory bowel disease (IBD) also seem to have a greater chance of acquiring CDAD (32). IBD has

been associated with dysbiosis, even without the use of antibiotics. This dysbiosis of the gut microbiota is likely to lead to an increased risk of CDAD. Indeed, in one case in an institution in Wisconsin, it was reported that the rate of CDAD in IBD patients rose from 7% to 16% over the space of a year from 2004-2005 (33). The association between CDAD and IBD is predominantly seen in ulcerative colitis patients and is not associated with small intestine Crohn's disease. *C. difficile* toxins may worsen the inflammatory process in patients suffering from ulcerative colitis. A greater mortality rate is associated with CDAD in patients who have IBD compared to patients with CDAD or IBD alone (34). The fact that some patients with IBD take immunosuppressive medications may also contribute to their risk of acquiring CDAD. It is also noteworthy that CDAD has been reported in perfectly healthy individuals without any underlying conditions and who have not taken antibiotics (35).

C. difficile colonization

C. difficile attaches to the mucus layer on the surface of enterocytes and penetrates the mucus layer using flagellae and proteases, such as the cysteine protease Cwp84 (36, 37). FliC is a flagellin monomer and FliD is the flagellar cap protein, involved in the attachment of *C. difficile* to intestinal mucus whereas Cwp84 is a cysteine protease which is responsible for degrading various extracellular matrix proteins such as vitronectin, fibronectin and laminin, helping *C. difficile* to penetrate the mucus layer of enterocytes (38-40). Interestingly, a study with flagella mutant isolates of the strain *C. difficile* 630 Δ*erm* showed that flagella mutant strains are

actually more virulent than the wild type strain in hamster models (41).This contradictory evidence to previous studies implies that flagella may not be crucial in mediating pathogenicity. On the contrary, repressed motility may actually be a virulence factor used by *C. difficile* (41). Using strain *C. difficile* 630, Aubry et al. demonstrated that the flagellar regulon in *C. difficile* is likely to be involved in modulation of toxin production and consequently virulence potential of the strain (42). Despite this, the precise involvement of *C. difficile* flagellae in colonization and pathogenesis remain largely unclear however. Interestingly, in a recent study, Jarchum et al. noted that administration of a flagellin derived from *Salmonella*, which is a toll-like receptor 5 (TLR5) agonist conferred protection against *C. difficile* colitis in a murine model of infection. The stimulation of TLR5 due to the flagellin led to decreased epithelial cell apoptosis and consequently led to an improved epithelial barrier function during *C. difficile* infection (43).

Degradation of host extracellular matrix proteins mediated by the above-mentioned Cwp84 protein enables the dissemination of *C. difficile* infection (37). Following the initial breach of the mucus layer of enterocytes, attachment of *C. difficile* to enterocytes occurs via multiple adhesins such as surface layer proteins (S-layer P36 and P47), a 68kDa fibronectin binding protein (Fbp68) and a cell wall protein (Cwp66)(39, 44, 45). A recent study has also highlighted the role of Surface Layer Protein A (SlpA) in mediating attachment of *C. difficile* to enterocytes (46). It was established that different domains of the SlpA protein are involved in this attachment process. Interestingly, it was also found that preparations consisting of SlpA from non-toxigenic strains disrupted the adherence of epidemic-associated *C. difficile* strains. Thus SlpA may be a potential target for preventing the attachment of *C. difficile* to host cells (46). In addition to the above-mentioned adhesion factors,

microtubule-based cell extensions mediate adhesion of binary toxin-producing strains of *C. difficile* (47, 48). Such protrusions have thus far only been demonstrated in strains producing binary toxin (47, 48).

Antibiotic resistance as a virulence factor

A significant factor contributing to *C. difficile* virulence is its resistance to several common antibiotics. Over time, *C. difficile* has developed resistance to antibiotics such as clindamycin. More recently, emergence of resistance among *C. difficile* strains to fluoroquinolones has been a grave concern, in particular amongst ribotypes 001, 106 and 027 (49, 50). In the majority of cases, antibiotic resistance in *C. difficile* strains has been acquired via mobilization of transposons, examples of which include the erythromycin resistance gene *ermB* on Tn5398, the tetracycline resistance gene on Tn5397, and the chloramphenicol resistance on Tn4453 (51, 52). Antibiotic resistance can also be attributed to point mutations in *C. difficile* e.g. a point mutation in the *gyrB* gene may be responsible for fluoroquinolone resistance (53). Drudy and co-workers also noted that certain *C. difficile* isolates which are erythromycin-resistant due to *ermB* also exhibit clindamycin resistance (54). A 23S ribosomal RNA methylase mediates resistance of some *C. difficile* strains to erythromycin (55). PCR ribotypes 006 and 001 exhibit increased resistance to erythromycin, along with levofloxacin and imipenem (56). Such resistance may account for the relatively high prevalence of such strains.

The chances of acquiring CDAD after antimicrobial therapy rise if the *C. difficile* strain is already resistant to the offending antibiotic i.e. that being used to treat the patient for the original unrelated condition (50). Indeed, a clindamycin-resistant *C. difficile* strain was responsible for a CDAD outbreak in various hospitals in the US in the 1990s (55). Also, the emergence and dissemination of outbreak-associated *C. difficile* ribotype 027 strains may be due to the widespread use of fluoroquinolone in clinical settings, which may be behaving as a selective pressure for these epidemic-associated R027 strains which are resistant to fluoroquinolone (27). In an *in vitro* study conducted in Spain, approximately 6% of *C. difficile* strains were found to be resistant to metronidazole and 3% of strains displayed intermediate resistance to vancomycin (57).

Spores

Another factor which contributes to *C. difficile* virulence is its ability to sporulate. Such spores are resistant to extremes of temperature, desiccation and exposure to various chemicals and can survive for months (58, 59). The contamination of environmental surfaces in hospitals and other health-care settings with *C. difficile* spores is a serious concern. Epidemic-associated *C. difficile* strains have been reported to be more capable of sporulating than non-epidemic strains *in vitro* (60-62). *In vitro* studies reported that the use of non-chlorine-based disinfectants at concentrations lower than the MIC actually encouraged sporulation to occur (60). Although other disinfectants inhibited *C. difficile* growth *in vitro*, chlorine-based disinfectants were the only ones effective against *C. difficile* spores (60). It is apparent that stringent infection control measures are of paramount importance in

hospitals. Isolating CDAD patients in private rooms and the use of gloves and gowns by clinicians have proved to be effective. Hand washing with soap and water is recommended to remove *C. difficile* spores whereas the use of alcohol hand gels is discouraged in clinical settings where there is an outbreak of CDAD and consequently an inherent risk of spreading spores, according to the Society for Healthcare Epidemiology (SHEA), Infectious Disease Society of America (IDSA) and the Centre for Disease Control (CDC) guidelines. The use of disposable thermometers instead of electronic thermometers can also reduce the likelihood of disseminating *C. difficile* spores (63). It is also worth noting that the use of some antibiotics can stimulate the germination of quiescent *C. difficile* spores which are present in the gut. This germination is followed by toxin production, which is most prolific in the late exponential phase (24, 64). The vancomycin derivative, oritavancin, has been shown to successfully inhibit the germination of *C. difficile* spores (65).

Paracresol production as a virulence factor

C. difficile produces a compound called para-cresol and it has been postulated that para-cresol may actually inhibit the re-colonization of the gastrointestinal tract by the commensal microbiota, even after antibiotic treatment has been discontinued (66). Para-cresol possesses bacteriostatic characteristics, and its production is through the transamination of tyrosine to para-hydroxyphenylacetic acid. This para-hyroxyphenylacetic acid is subsequently decarboxylated to para-cresol. Paracresol can also inhibit other *C. difficile* strains. It is noteworthy in this regard that an

outbreak-associated R027 strain isolated in Stoke Mandeville (20291 R027) is able to tolerate para-cresol better than a previously isolated CD196 R027 strain (66).

PATHOGENESIS

Clinical presentation

Several factors are required for the development of CDAD. Sorg & Sonenshein reported that the germination of *C. difficile* spores is aided by the presence of bile salts (18). Once germination has occurred, vegetative cells begin to release toxin A and toxin B, possibly triggered by a change in the availability of certain amino acids (18). Both Toxin A and toxin B display enterotoxic and cytotoxic activity, causing inflammation, fluid secretion and tissue necrosis of the intestinal epithelium (67-70). Typically, CDAD presents as watery profuse diarrhoea 48-72 hours post-infection. Clinical signs of CDAD include new-onset diarrhoea, abdominal distension and leukocytosis (71). CDAD affects the colon and the presence of colonic pseudomembranes by endoscopic examination is a common sign. Amongst the risk factors for the development of an aggressive form of *C. difficile* colitis include: underlying malignancy, immunosuppressive medications such as corticosteroids, patients on anti-peristaltic medication and patients being treated with clindamycin (72, 73). Patients with aggressive colitis have a higher chance of presenting with peritonitis, hypoalbuminaemia and an abnormal white blood cell count (74).

Expeditious diagnostic and treatment modalities are imperative for such a group of patients with underlying immunosuppression or illnesses.

C. difficile toxins cause ulcers to develop on the intestinal mucosa, which in turn leads to the release of mucus, inflammatory cells and serum proteins, with the appearance of pseudomembranes. Pseudomembranes due to CDAD typically appear as raised yellow or white plaques approximately 2cm in size, found scattered over the colorectal mucosa (75). *C. difficile* colitis progresses to fulminant colitis in approximately 3-8% of cases (76). The signs of fulminant colitis include diffuse abdominal pain, fever, abdominal distension, lactic acidosis and significant leukocytosis. Further complications may include toxic megacolon and bowel perforation (74). Patients presenting with diarrhoea generally have stools which are unformed, watery and mucoid in nature. Abdominal pain, raised temperature and leukocytosis accompanied by diarrhoea are perhaps the most common symptoms (71). Despite these common signs, it must be highlighted that the clinical appearance of CDAD varies greatly amongst patients and can range from asymptomatic patients to patients presenting with mild diarrhoea to severe PMC and toxic megacolon (77, 78).

***C. difficile* toxins**

As noted above, the two toxins that are primarily associated with *C. difficile* pathogenesis are toxins A and B. Toxin A (TcdA) is a 308kD toxin and toxin B (TcdB) has a mass of 270kD (79, 80). Their mode of action involves the glucosylation of GTPases Rho, Cdc42 and Rac, thereby causing impaired actin polymerization and leading to cytoskeletal disorganisation in epithelial cells (81, 82).

As mentioned, both TcdA and TcdB are large cytotoxins which cause significant epithelial tissue damage and colonic inflammation (67-69,83). The end result of this tissue damage and colonic inflammation is diarrhoea as a result of fluid loss into the lumen of the intestine. Both toxins A and B belong to a group of large clostridial toxins (LCT) (84, 85). Other members of the group include TcsL and TcsH from *Clostridium sordellii*, TpeL from certain *Clostridium perfringens* isolates and the α-toxin (TcnA) from *Clostridium novyi* (84, 85). Like TcdA and TcdB, other members of this group of toxins are monoglycosyltransferases that trigger the inactivation of the Rho family of GTPases, thereby rendering them inactive and thus repressing downstream cellular activities (84, 85). The C-terminus of LCT binds to a receptor(s) on the target cell surface. The precise characteristics of the toxin receptors remain unclear, with protein, carbohydrate or glycolipid receptors all plausible (86). Endocytosis of the toxins occurs through clathrin and dynamin-mediated pathways, making their way into the endosomes of the target cell (87). Translocation of the toxins from the endosomes into the target cell cytosol is thought to involve endosome acidification. Indeed, the macrolide bafilomycin A1, which represses endosomal acidification via inhibition of the endosomal vacuolar ATPase pump, represses toxin B-mediated cytotoxicity (88). A conformational change of both toxins A and B takes place, which is induced by a change of pH within the endosome. This change of shape likely occurs between the cysteine protease domain and the C-terminal combined repetitive oligopeptide (CROP) region (86, 89, 90). A protrusion begins to form, putatively involved in membrane interaction. Toxin A forms pores at acidic pH, with cholesterol required for toxin A-induced pore formation. The hydrophobic region of toxin B, especially glutamate 970 and 976, is thought to be important in toxin B-mediated pore formation. Glutamate residues at

positions 970 and 976 serve as pH sensors for membrane interaction (86). After pore formation, a change of conformation within the cysteine protease domain of the toxin is triggered by binding of inositol hexakisphosphate. This in turn leads to cleavage between the cysteine protease domain and glucosyltransferase domain, allowing the N-terminal glucosyltransferase domain to escape into the cytosol.

Toxin A and toxin B-mediated inactivation of Rho GTPases has a myriad of negative effects on the target cell. Actin depolymerisation is elicited and thus structural integrity of the cell is compromised. The inactivation of Rho GTPases by these toxins also affects the signalling and motility of immune cells as well as compromising epithelial barrier function. The loss of the cell's structural integrity eventually leads to caspase-3 and caspase-9-mediated apoptosis (91, 92). This in turn results in impaired tight junctions between cells and further compromised epithelial barrier function. Cytokines are released by the epithelial cells upon exposure to the toxins, resulting in activation of mast cells, neutrophils and other cells (91-93). The further release of pro-inflammatory cytokines and neuropeptides also compromises the integrity of the epithelial barriers and tight junctions, resulting in elevated permeability and accumulation of fluid in the intestine, the manifestation of which is diarrhoea (94).

Although for decades it was thought that toxin A was the primary virulence factor, more recent studies have indicated the presence of toxin B-positive, toxin A-negative strains in patients with CDAD (53). Lyras et al. reported that isogenic mutants of *C. difficile* which lacked the toxin B gene (*tcdB*) exhibited reduced virulence in rodent models of CDAD, thus supporting the theory that toxin B is indeed an important virulence factor (95). The altered substrate specificities of variant toxin B, produced by toxin A^-B^+ strains may play an important role in causing more severe disease (53,

78, 96). Furthermore, the lack of a toxin A-specific host immune response may also result in more severe symptoms (97). A recent study has demonstrated that cell death elicited by toxin B is dependent on the assembly of host epithelial cell nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex, while reactive oxygen species (ROS) also play a role. It was shown in the study that diphenyleneiodonium (DPI) and/or N-acetylcysteine (NAC) prevented colonic tissue damage by toxin B. The mechanisms for this involved a decrease in ROS-mediated tissue damage and may have the potential for reducing necrosis of colonic mucosa in CDAD patients (98). The host SLC11A1 gene has also been shown to be involved in the pathogenicity caused by toxin B. Upregulation of the gene caused by toxin B results in Rho GTPase glucosylation and consequent tissue necrosis (99).

Certain *C. difficile* strains, including R027 isolates, produce a binary toxin in addition to toxin A and toxin B. Despite this, a definitive link between specific PCR ribotypes and binary toxin production remains unclear (93, 100). It has been postulated that binary toxin acts in conjunction with TcdA and TcdB in the pathogenesis of both 'hypervirulent' and virulent isolates (93, 101). This binary toxin is an actin-specific adenosine diphosphate (ADP) ribosyltransferase (102). Other toxins belonging to this subgroup of two-component ADP ribosyltransferases in the genus *Clostridium* include the *C. botulinum* C2 toxin and the *C. perfringens* iota toxin (103, 104). The function of binary toxin has not been clearly elucidated. Binary toxin-positive, toxin A^-B^- strains of *C. difficile* are not pathogenic in hamsters (101). Nonetheless, in a rabbit ileal loop test, the cell-free supernatant from such strains was shown to result in fluid secretion (101).

Pathogenicity locus (PaLoc) and *C. difficile* toxin production

Until recently, investigating the role of *C. difficile* genes had been problematic. However, advancements in targeted mutagenesis systems have helped this cause (93, 105, 106). A 19.6 kb pathogenicity locus (PaLoc) encodes the genes for these toxins (*tcdA* and *tcdB*) along with genes for putative positive and negative regulators of toxin expression (*tcdR* and *tcdC* respectively), as well as *tcdE*, encoding a putative holin protein (Fig. 1A)(107, 108). TcdR functions as an alternative RNA polymerase sigma factor, and thus behaves as a positive regulator of toxin gene expression (109). In contrast, TcdC was initially thought to serve as a negative regulator of toxin production, destabilising the TcdR-holoenzyme, thus hindering transcription of the PaLoc (107). However, in recent times, it has been demonstrated that transcription levels of the genes in the PaLoc and consequent total toxin production barely differs between a wild type *C. difficile* 630 Δ*erm* strain and its *tcdC* mutant, suggesting that TcdC may not be a key regulator of toxin expression in the strain (110). It is noteworthy that earlier studies investigating the role of TcdC were *in vitro* investigations (111, 112). The *in vivo* mechanisms of this protein had remained largely unclear however. A recent *in vivo* study has now indicated that TcdC may not actually play a key role in *C. difficile* virulence (113). Furthermore, in another recent study, it was noted that there was no decrease in *tcdC* expression levels during stationary phase of growth, implying that TcdC may serve a modulatory function instead of a previously-hypothesised repressive function (61). Polymorphisms in the *tcdR-tcdB* intergenic region as well as in the *tcdR* ribosome binding site (RBS) in the 'non-hypervirulent' VPI 10463 strain (which still produces high levels of toxins) likely results in increased translation of TcdR, consequently leading to read-through transcription of the toxin genes. Such polymorphisms may account for the increased

levels of toxins produced by some isolates (61). In addition, the study found that epidemic-associated strains sporulated at an earlier stage and produced a greater number of spores than other non-epidemic-associated isolates. Thus, increased sporulation rates along with high level toxin production may explain the outbreak-associated nature of such hypervirulent strains (61). In an *in vitro* study, Vohra & Poxton noted that outbreak-associated R027 strains produced higher amounts of toxins in the logarithmic and stationary growth phases, compared to other ribotypes (62). Moreover, epidemic-associated strains were found to produce more toxins and a greater number of spores relative to R012 (62). It was also particularly noteworthy that *tcdC* expression levels were not attenuated during stationary growth phase, as was previously thought, lending credence to the novel hypothesis that TcdC has a modulatory effect on toxin production, instead of a repressive one. In addition, an elevated level of expression of *tcdE* in R027 strains highlights its involvement in the release of the toxins. Therefore, it is hypothesised that a combination of factors, including greater toxin production, increased spore formation and increased expression of holin proteins may contribute to the epidemic-associated traits of certain strains (62). Interestingly however, using isogenic strains of *C. difficile*, Carter et al. showed that a naturally occurring mutation in *tcdC* is responsible for the hypervirulence of epidemic *C. difficile* isolates (111). Thus, the precise mechanisms of action of TcdC *in vivo* have yet to be definitely ascertained.

For a long time, the elucidation of the precise functions of TcdA and TcdB using a genetic approach was hampered by a lack of tools to isolate *C. difficile* isogenic toxin gene mutants. However, Lyras and co-workers as well as Kuehne and co-workers facilitated the study of isogenic mutants (95, 114). Syrian golden hamsters were infected with either toxin A^- mutants, toxin B^- mutants or wild type strains in a

study (95). Infection with the wild type strain resulted in the death of 90% of hamsters included in the study. Infection with toxin A^- mutants resulted in death of 94% of hamsters, indicating that toxin A is not crucial for disease. Interestingly, infection with toxin B^- strains only caused disease in 22% of hamsters, highlighting that toxin B was in fact the main virulence factor rather than toxin A, contrary to previous assumptions. More significantly, the study helped to explain the pathogenicity caused by toxin A^-B^+ isolates in clinical settings (95).

Importantly, the genes encoding binary toxin, designated *cdtA* and *cdtB* are not part of the PaLoc but are nevertheless found on the chromosome (Fig. 1B) (93, 101, 102, 108). CdtR is a response regulator encoded by the binary toxin locus (CdtLoc) and controls binary toxin production (93).Isolates which do not produce binary toxin have a conserved 68bp sequence, in place of the CdtLoc (93).

Diagnostics

An early and accurate diagnosis of CDAD is highly important. Currently, the most popular tests rely on the detection of toxins A and B by polymerase chain reaction (PCR), nucleic acid amplification tests (NAATs) and/or anaerobic culture of isolated *C. difficile* strains, cytotoxin assays and immunoassays. Various typing systems have been employed to discriminate between strains. Examples include restriction endonuclease analysis (REA), pulsed-field gel electrophoresis (PFGE), PCR ribotyping and toxinotyping. A list summarising the main diagnostic and typing methods used for *C. difficile* is included in Table 1.

Variant toxins and toxinotypes

C. difficile exhibits significant chromosomal variations between different strains, including differences in the PaLoc and *tcdA* and *tcdB* genes (115-117). This is the basis for *C. difficile* toxinotyping systems, in which strains are classified into variant toxinotypes according to restriction fragment length polymorphism-PCR (RFLP-PCR) analysis of toxin genes (96, 117). These toxinotypes take into account specific mutations/changes within the toxin genes (96). Any such mutations are compared against the toxinotype 0 reference strain VPI 10463. To date, there are approximately 30 *C. difficile* toxinotypes identified (78, 118). Mutations within the PaLoc can lead to a loss of production of one or indeed both of the *C. difficile* toxins. Most toxin A^-B^+ strains belong to toxinotypes VIII, X, XXX and XXXI whereas toxin A^-B^-strains belong to toxinotype XIa and XIb (96, 117-119). Such variant toxin strains are likely to have specific substrate targets as well. The toxin B protein from toxinotype VIII, X and XIV acts by inactivating a different set of Rho GTPases. Toxin B produced by toxinotypes X and VIII inactivates Rap, Ral and R-Ras GTPsases by glucosylation, whereas toxin B from *C. difficile* VPI 10463 (toxinotype 0) inactivate Rho, Rac and Cdc42 by glucosylation (96, 117). In a mouse model, the lethal dose of toxin B from a toxinotype X strain was found to be 8 times less than the lethal dose for the toxinotype 0 toxin (108).The toxin B from an outbreak-associated strain 20291 R027 (toxinotype III) was compared to toxin B from *C. difficile* 630 (toxinotype 0)(109). A significant variation was noted in the C-terminal receptor binding domains of the toxins, with less than 80% identity in some regions of the C-terminus. Moreover, in a zebrafish model, the entry of purified toxin B from an outbreak-associated strain into target cells was shown to be much quicker than the toxin B from VPI 10463 strains, causing a greater degree of tissue destruction and necrosis (109). It is plausible that

the variations in the receptor binding domain of toxin BHV from 'hypervirulent strains' allow it to bind to multiple binding sites/receptors, compared to toxin B from 'non-hypervirulent' strains. This mechanism of binding of toxins to widely distributed receptors may account for the epidemic-associated properties of certain strains. Non-toxigenic *C. difficile* strains have also been isolated from asymptomatic patients. These strains do not contain the toxin A and toxin B genes (120, 121).It has been proposed that non-toxigenic strains have the potential to inhibit infection and recurrence of infection by competing with toxigenic strains for adherence in the intestine and for nutrients (120, 121). A recent study with hamster models of infection has further highlighted the potential benefits of non-toxigenic *C. difficile* (122). The introduction of non-toxigenic *C. difficile* has been shown to cause remission of infection in two patients (123). Research is ongoing investigating the possibility of using non-toxigenic *C. difficile* as a potential prophylactic for CDAD.

A list of *C. difficile* toxinotypes and toxin-production characteristics is included in Table 2.

R027 strains

Several ribotypes of *C. difficile*, including the notorious R027 strains, have caused outbreaks in Europe and North America over the last decade. *C. difficile* ribotyping is a typing method involving the fingerprinting of *C. difficile* genomic DNA restriction fragments using specific restriction enzymes. The process involves amplifying specific fragments of the 16S and 23S spacer regions by PCR and digesting the fragments with specific restriction enzymes in order to compare the profiles from different strains (124). Studies have shown that PCR R027 causes more

severe diarrhoea, along with higher recurrence rates and mortality rates (125, 126). The increased severity of disease caused by 'hypervirulent' R027 strains is likely to be due to the production of higher amounts of toxin compared to other ribotypes (61, 62). Factors other than toxin production may also account for R027 'hypervirulence,' such as enhanced tolerance to bile salts, increased germination of spores and increased dissemination due to sporulation. Akerlund and co-workers reported that an epidemic R027 strain had enhanced sporulation capabilities relative to three other non-epidemic R027 isolates (127). The 3'end of the *tcdB* gene, which encodes the binding domain of toxin B, has been shown to be variable in R027 strains, implying that the binding capacity of toxin B from R027 may be distinct from other less virulent isolates (128).

C. difficile resistance to cationic antimicrobial peptides (CAMPs)

In order to survive and proliferate in the gastrointestinal tract (GIT), *C. difficile* must contend with cationic antimicrobial peptides (CAMPs) produced by the human host and other bacteria. McBride & Sonenshein found that upon low level exposure to cationic antimicrobial peptides, *C. difficile* expresses genes involved in CAMP resistance (129). The authors discovered that an operon encoding an ATP-binding cassette (ABC) transporter and an orphan histidine kinase gene adjacent to the operon are involved in mediating resistance to CAMPs in *C. difficile* (129). The genes were designated *cprABC* and *cprK* respectively and it was subsequently discovered that a non-contiguous two component system (*cprK* and *cprR*) was involved in mediating resistance to CAMPs (130). In a separate study, it was noted that the *dlt* operon also mediates resistance to CAMPs in *C. difficile* (131). The *dlt*

operon is involved in adding d-alanine to teichoic acid, leading to an overall positive charge on the bacterial cell surface, which in turn confers protection against similarly charged CAMPs (131). In another study, McQuade et al. determined that epidemic-associated *C. difficile* R027 strains exhibited increased resistance to the CAMP cathelicidin LL-37 compared to non-epidemic isolates (132).An inducible system involved in resistance to CAMPs has been suggested, as exposure of *C. difficile* to sub-inhibitory LL-37 concentrations led to an even further increase in resistance (132). Alterations in amounts of surface layer proteins, ABC transporters, cell wall synthesis proteins, as well as proteins involved in lysine metabolism were particularly noteworthy in *C. difficile* strains in response to cathelicidin LL-37 in the study (132).

THERAPEUTIC OPTIONS

Current treatment of CDAD

The current treatment modalities for CDAD involve the immediate discontinuation of antibiotics given to the patient for other diseases, and commencement of metronidazole and vancomycin administration post-haste. The rates of metronidazole treatment failure are significantly higher in patients who are still on other antibiotics, due to continued perturbations of the competing gut microbiota (133).One study suggested that metronidazole and vancomycin were equally effective for mild cases of CDAD, with treatment success rates of 90% and 98% respectively

(134).However, for more severe cases, vancomycin was the treatment of choice, as success rates for metronidazole and vancomycin were 76% and 97% respectively, though recurrence rates were similar i.e. 15% of metronidazole-treated patients, compared to 14% for vancomycin (134).In this regard, it must be noted that slightly different success rates are reported for metronidazole and vancomycin against *C. difficile*, depending on a variety of factors, such as type of study conducted, sample size and geographical location. High failure rates for metronidazole, due to the emergence of the outbreak-associated R027 strains and also a rise in the number of elderly patients in hospitals affected by CDAD who are already being treated with other antibiotics, have also been reported (135, 136). The treatment for non-epidemic and epidemic *C. difficile* strains appears to be similar, with metronidazole as the primary treatment choice for mild-moderate cases of CDAD, followed by vancomycin for more severe CDAD, according to the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) guidelines. Fidaxomicin has already been proved to have promising efficacy as a therapeutic for CDAD. Other antibiotics such as ramoplanin, tigecycline and the rifamycin group have shown potent activity against *C. difficile* and research is ongoing regarding their clinical efficacy against CDAD. Fidaxomicin and faecal microbiota transplantation (FMT) is also strongly advocated by ESCMID for recurrent CDAD cases (137, 138).

For mild-moderate CDAD, 500mg of oral metronidazole three times a day for 10-14 days is recommended, whereas 125mg of oral vancomycin four times a day for the same duration is indicated for more severe cases of CDAD (139).Oral vancomycin, supplemented with intravenous metronidazole if necessary, is recommended for severe CDAD by the IDSA. Although oral administration of metronidazole or vancomycin is optimal, metronidazole can also be administered intravenously as it is

capable of reaching the intestinal lumen via diffusion across the inflamed colon. A prospective study conducted by Wenisch et al. demonstrated that oral metronidazole (7.4% mortality) was more effective against *C. difficile* than intravenous metronidazole (38.1% mortality)(140). Vancomycin may also be introduced intracolonically or as a retention enema. 100mg of the glycopeptide teicoplanin twice a day in addition to metronidazole or vancomycin is recommended by the ESCMID for CDAD (141).However, fusidic acid and bacitracin do not seem to be as effective as glycopeptides or metronidazole and thus are contraindicated by ESCMID. Fusidic acid, oral bacitracin and teicoplanin are not recommended in the USA for CDAD (141).

Alternative vancomycin dosing strategies

Though vancomycin and metronidazole have been used for the treatment of CDAD for the last three decades, recurrence and relapse of disease still remains a serious problem. About 50% of cases of recurrence of disease are due to relapse, whereby the original *C. difficile* strain that was culpable for the infection causes symptoms again, due to spores surviving the CDAD treatment (142).

Alternative dosing regimens for vancomycin have been investigated to circumvent the problem of recurrent CDAD. Pulsed vancomycin dosing, which involves short intermittent administration of vancomycin, as well as tapered dosing of vancomycin have proved successful. In a retrospective study conducted by McFarland et al. with a total of 163 patients, a subset received vancomycin and a smaller subset received tapered doses of vancomycin, whereby vancomycin was decreased incrementally from (500mg-3g daily) – (125-750mg daily) over 3 weeks (143). A 31% recurrence

rate was noted for this tapered dose strategy compared to recurrence rates of 43-54% with conventional vancomycin dosing for 10 days. Pulsed vancomycin administration of 125-500mg per day every 2-3 days for 27 days resulted in a 14% recurrence rate (143). Although no randomized controlled trials have assessed such dosing regimens, the IDSA and the SHEA recommend the use of pulsed or tapered vancomycin dosing for second or third recurrences of CDAD (139).In cases of extremely severe *C. difficile* colitis, intracolonic vancomycin administration may have the potential to be used as an adjunct, to ensure higher concentrations of the drug in the colon (144, 145). This may involve the use of an intracolonic bolus with an intravenous solution of vancomycin. A rectal retention enema such as 500mg of vancomycin in 1L of saline solution may be another option. Kim et al. reported a 70% success rate in a recent trial whereas Apisarnthanarak et al. approximated a success rate of 57-75% for intracolonic vancomycin administration in a review of relevant case series (144, 145).

Fidaxomicin

Fidaxomicin is an oral macrocyclic antibiotic, produced by *Dactylosporangium aurantiacum*, targeted against *C. difficile* (146). One of the main advantages of fidaxomicin is that it is tailored specifically towards *C. difficile*, with little impact on the commensal gut microbiota and has been shown to inhibit spore formation and toxin production in *C. difficile* (137, 147, 148). This narrow spectrum of action is hugely beneficial as it permits quick restoration of the commensal gut microbiota in CDAD patients, and thus decreases the risk of recurrence of disease due to overgrowth of *C. difficile* (137, 148). Another advantage is that it sustains a certain

level of antibacterial activity for a more prolonged period, compared to metronidazole and vancomycin (146). Therefore, it is capable of inhibiting *C. difficile* at concentrations lower than the MIC.

A number of *in vitro* and *in vivo* studies have highlighted the potential of fidaxomicin in combating *C. difficile*. It is promising to note that fidaxomicin has potent antimicrobial activity against outbreak-associated R027 strains as well as non-epidemic-associated strains (149-151). Phase I clinical trials conducted by Shue et al. reported low plasma concentrations of fidaxomicin of less than or equal to 5ng/ml, with a concomitant near 100% recovery of fidaxomicin and its metabolite in faeces (152). These findings were in accordance with more recent Phase II and Phase III trials which reported that the concentrations of fidaxomicin in faeces were 2000-10000-fold higher than the MIC_{90} value against *C. difficile.*

Clinical trials with CDAD patients have shown that fidaxomicin caused fewer recurrences and thus, is indicated for mild, moderate, severe and recurrent CDAD (153, 154). A Phase II trial by Louie and co-workers assessed the efficacy of several doses of fidaxomicin in treating CDAD (155). Response rates of 71%, 80% and 94% were found for patients treated with 100mg, 200mg and 400mg of fidaxomicin respectively. Four patients receiving either 100mg or 200mg of fidaxomicin failed therapy, representing an 8.9% failure rate. Two out of 41 patients in the study exhibited recurrence of disease a month after treatment (155). In two phase III clinical trials with 1105 CDAD patients, treatment with fidaxomicin resulted in comparable initial response rates to vancomycin.(156). Also, the group of patients treated with fidaxomicin had a 47% lower recurrence rate compared to vancomycin (156). In patients with recurrent CDAD, 35.5% of 128 patients receiving vancomycin had a further recurrence whereas only 19.7% had a further recurrence

when treated with fidaxomicin as reported in two studies (149, 150). Although lower recurrence rates have been observed for fidaxomicin compared to vancomycin, the recurrence rates are broadly similar for R027 strains (137, 149, 150).

Rifamycin antibiotics

The rifamycin subgroup of antibiotics, which include rifaximin, rifampin, rifalazil and others display potent anti-*C. difficile* activity *in vitro* (157, 158).However, only a handful of reports regarding their clinical use are available (159). In one instance it was reported that 7 patients recovered from CDAD with 3 days of rifampin treatment. The dose was 300-600 mg rifampin administered every 12 hours in combination with vancomycin (160). Another study evaluated the effects of metronidazole and rifampin combination therapy, but no beneficial effects were noted compared to metronidazole treatment alone (161). Rifaximin, another rifamycin antibiotic, also shows strong antimicrobial activity against *C. difficile in vitro* (157). The rates of *C. difficile* resistance to rifaximin are significantly lower compared to rifampin (159). In an *in vitro* study, O'Connor and co-workers found that 14 *C. difficile* isolates were resistant to rifaximin, out of 80 strains tested in the study whereas rates of metronidazole resistance amongst *C. difficile* strains have been reported to vary from 15-35% in a separate study (162, 163). Rifaximin resistance has been associated with mutations in the *rpoB* gene.

A clinical trial conducted by Johnson et al. showed that 7 of 8 patients administered rifaximin following vancomycin displayed no recurrence of CDAD (164). Rifaximin was effective in eradicating symptoms of diarrhoea in three liver transplant patients, within 36-48 hours of administration (158). A success rate of 64% for rifaximin was

reported by Patrick-Basu and co-workers in 25 patients who failed metronidazole treatment (165).

Nitazoxanide

Nitazoxanide is a nitrothiazole benzamide which displays potent antimicrobial activity against intestinal parasites and GIT pathogens including *C. difficile* (166).Musher et al. conducted a randomized, prospective double blind study with hospitalised patients with *C. difficile* colitis. Patients included in the study were those who had primary CDAD (a minimum of 3 unformed stools per day), with symptoms such as abdominal pain, fever or leukocytosis and an enzyme immunoassay indicating *C. difficile* infection(167).89.5% of CDAD patients responded to nitazoxanide therapy, which was better than the 82.4% response rate for metronidazole, after a week of therapy in the trial. Furthermore, the sustained response to nitazoxanide a month after therapy was comparable to metronidazole rates (167).The same authors further investigated the efficacy of nitazoxanide in treating CDAD patients who had failed metronidazole treatment. Initially, a 74% response rate for nitazoxanide was noted with this patient group. However, subsequent recurrence of disease resulted in a final cure rate of 54% for nitazoxanide in treating CDAD patients not responding to metronidazole (168).Another recent prospective double blind randomized study in 2009, also conducted by Musher et al. compared the efficacy of 10 days of nitazoxanide therapy versus 10 days of vancomycin therapy with 50 CDAD patients. Patients included in the study were those who had confirmed positive tests for *C. difficile* toxins, had more than three unformed stools within a 24 hour period and presented with at least one of the

following: abdominal pain, fever or leukocytosis (169). Response rates of 77% for nitazoxanide and 74% for vancomycin were noted initially. Initial response rates in the study were defined as the absence of any CDAD symptoms between days 11-13 (169). Amongst the patient group who completed nitazoxanide and vancomycin therapy, 94% and 87% final response rates were noted respectively. One patient treated with nitazoxanide and 2 patients treated with vancomycin displayed relapse of disease. Although a small sample size was used in the study, acknowledged by the investigators, the findings of the study led to the conclusion that nitazoxanide was comparable to vancomycin in terms of treating CDAD (169).

Tigecycline

Tigecycline, a derivative of minocycline, is a drug which undergoes very little metabolism, resulting in a large percentage of the active compound being excreted in the faeces (170). In a study by Baines et al. using a human gut model, tigecycline prevented the growth of *C. difficile* and consequent toxin production (171).Similar observations were made by Jump et al. studying tigecycline using a mouse model of infection (172). A few case reports have described the success of tigecycline in combination with other antibiotics, in treating CDAD in patients failing conventional metronidazole and vancomycin therapy (173, 174) while another study has highlighted the success of tigecycline on its own in resolving CDAD (170). One report however stated that tigecycline administration for as long as 14 days still failed to treat a case of CDAD (175).Despite a few case reports highlighting the success of tigecycline and rifaximin, the guidelines drafted by the SHEA/IDSA in

2010 do not include tigecycline, rifaximin or linezolid as part of CDAD therapeutic options (139).

Ramoplanin

Ramoplanin is a lipoglycodepsipeptide antibiotic which was developed as an oral agent for use in patients colonized with vancomycin-resistant enterococci but also exhibits potent anti-*C. difficile* activity mediated through the inhibition of cell wall synthesis (25, 176).Using a hamster model of *C. difficile*-induced colitis, Jabes and co-workers reported that ramoplanin was a better treatment choice than vancomycin, while in a separate study, Freeman and co-workers found the efficacy of ramoplanin to be comparable to vancomycin in hamster models of infection (177, 25).' The study by Freeman et al. showed that administration of ramoplanin resulted in a resolution of symptoms in a hamster model of CDAD and a reduction in toxin titre in an *in vitro* gut model (25). The study also showed the superior efficacy of ramoplanin over vancomycin against *C. difficile* spores, as spores were recovered less often from the ramoplanin-treated hamsters, compared to vancomycin-treated hamsters (25). Doses of 200-400mg of ramoplanin administered twice a day for 10 days were effective and comparable to vancomycin for the treatment of CDAD, according to a phase II trial (178). Although ramoplanin is not yet used to treat CDAD, it may eventually become an alternative antibiotic of choice due to its potent anti-*C. difficile* activity.

Bacteriocins against *C. difficile*

Bacteriocins are ribosomally synthesised antimicrobial peptides with either narrow spectrum or broad spectrum activity against other bacteria (179). To date, the activity of a few bacteriocins has been assessed against *C. difficile*. Bacteriocins, due to their ribosomally-synthesised nature, can also be the subject of bioengineering strategies to find derivatives with ameliorated bioactivity against specific bacterial targets, such as *C. difficile*. Furthermore, some probiotic strains have the ability to produce bacteriocins *in situ*. Since bacteriocins are currently not used in the clinic against *C. difficile*, the development of resistance amongst target *C. difficile* strains has not been a problem thus far. When considering the use of bacteriocins as an alternative/adjunctive therapeutic option for CDAD, the mode of delivery of the bacteriocin to the colon must be carefully evaluated. Encapsulation of the bacteriocin may be a means to overcome proteases. It must be noted that the anti-*C. difficile* activities of the bacteriocins described herein are predominantly based on *in vitro* studies and the *in vivo* efficacies of the majority of these bacteriocins have yet to be determined.

Thuricin CD

Thuricin CD is a recently discovered bacteriocin with potent narrow spectrum activity against *C. difficile* (180). The main advantage of thuricin CD is that its antimicrobial activity is largely restricted to *C. difficile* and has little or no impact on other commensal gut microbes. This was demonstrated using a model of the human distal colon and a high-throughput sequencing approach which revealed that thuricin CD had minimal impact on the numbers of *Firmicutes*, *Bacteroidetes* and

Proteobacteria, compared to vancomycin and metronidazole which elicited a decrease in *Firmicutes* and *Bacteroidetes* numbers, concomitant with an increase in *Proteobacteria* numbers (181).

Nisin and lacticin 3147

Nisin is a member of the lantibiotic family of bacteriocins with broad spectrum antimicrobial activity against a range of Gram-positive bacteria, including antibiotic-resistant bacteria and food pathogens (182).Studies by Field et al. showed that a bioengineered derivative of nisin A, designated M21V, displayed more potent antimicrobial activity against a variety of Gram-positive pathogens, including *C. difficile* R027, compared to wild type nisin A (182). Lacticin 3147 is a two-peptide lantibiotic produced by *Lactococcus lactis* DPC 3147 (183).It has a broad spectrum of antimicrobial activity against Gram-positive pathogens. Lacticin 3147 seems to trigger a rapid lysis of log phase *C. difficile* cells, measured by quantifying the release of acetate kinase. Addition of high lacticin 3147 concentrations of 6 μg/ml results in the decrease of *C. difficile* ATCC 43593 cell numbers from 10^6 cfu/ml to zero in 2 hours. Subsequent studies with lacticin 3147 using a model of the human distal colon showed that it caused a decrease in *Firmicutes* numbers with a concomitant increase in *Proteobacteria* numbers (181).

Actagardine and NVB302

Actagardine A is a 19-amino acid lantibiotic with potent antimicrobial activity against Gram-positive bacteria, including *C. difficile*. A bioengineered V15F

derivative of actagardine A exhibited lower MIC values against the same *C. difficile* targets compared to the wild type actagardine (184). NVB302 is a semi-synthetic type B lantibiotic, derived from actagardine and is effective against *C. difficile*. Crowther et al. conducted a recent study investigating the efficacy of NVB302 compared to vancomycin in treating CDAD employing an *in vitro* gut model (185). The gut microbiota count as well as the *C. difficile* viable counts and spores were enumerated following NVB302 and vancomycin administration and a decrease in viable *C. difficile* counts with vancomycin and NVB302 administration was noted. NVB302 performed better than vancomycin as cytotoxin levels were undetectable 7 days subsequent to NVB302 administration compared to undetectable cytotoxin levels 5 days after vancomycin instillation. *C. difficile* spores did not germinate following either vancomycin or NVB302 instillation (185).

LFF571 (GE2270 derivative)

GE2270 is a thiopeptide bacteriocin which inhibits translation in bacteria (186).LFF571 is a semi-synthetic derivative of the thiopeptide GE2270, developed by Novartis, which displays antimicrobial activity against a range of Gram-positive bacteria, including *C. difficile* (187). The *in vivo* activity of LFF571 against *C. difficile* was compared with vancomycin in a study with Golden Syrian hamster models (188). LFF571 was administered orally 24 hours post-infection. Doses of 0.2, 1, 2, 5 or 10mg/kg of LFF571 were used. Administration of 5mg/kg LFF571 resulted in a 71% initial response rate, whereby 5 out of the 7 hamsters survived after 21 days, while 37.5% of animals survived 21 days when treated with 20mg/kg vancomycin. In terms of recurrence rates, LFF571 once again fared better than

vancomycin. Only 2.2% of hamsters had recurrence at the conclusion of treatment with 5mg/kg LFF571, whereas 37.8% of hamsters which survived at the termination of treatment with 20mg/kg vancomycin, experienced recurrence (188).

Recently, a randomized double blind trial was conducted, investigating the efficacy and safety of LFF571 in healthy volunteers (189).Encouragingly, no serious side effects of LFF571 were noted amongst 56 volunteers. LFF571 largely remained in the gut with very low concentrations noted in serum (the highest concentration being 3.2ng/ml in serum in one volunteer). Moreover, LFF571 was tolerated equally well irrespective of single or multiple doses in healthy volunteers participating in the study (189).

A summary of MIC values of antibiotics being investigated to replace metronidazole/vancomycin as well as various bacteriocins against *C. difficile*, as reported in published studies, is included in Table 3.

Faecal transplantation

Since the main risk factor for acquiring CDAD appears to be the perturbation of the gut microbiota due to broad spectrum antibiotics, and subsequent overgrowth of *C. difficile*, the restoration of the intestinal microbiota via faecal transplantation/faecal bacteriotherapy seems like an appropriate therapeutic option. Faecal bacteriotherapy is the process of introducing faeces from a healthy donor, in a liquid suspension, into the GIT of a patient (200). Typically, patients considered for faecal bacteriotherapy are those who have confirmed *C. difficile* colitis and have had at least two relapses following antibiotic therapy. Stool donors are screened for HIV-1, HIV-2, hepatitis

A, B, C and faecal samples tested for bacterial/parasitic pathogens such as *Salmonella*, *C. difficile*, *S. aureus* (200, 201).The majority of cases of faecal transplantation occur through the rectum, but nasogastric, nasoduodenal, nasojejunal instillations are common as well. 250mg of vancomycin every 8 hours for 4 days and two 20mg doses of omeprazole per day for 4 days are administered to the transplant recipient to decrease *C. difficile* numbers and allow the introduced bacteria to colonize by decreasing acid production in the stomach and consequently elevating the pH, in the event of nasogastric instillations (202-204). The treatment success rate of faecal bacteriotherapy for recurrent CDAD has been reported to be approximately 90% in a study involving 18 subjects, while a response rate of 100% was reported when 12 recurrent CDAD patients were treated with faecal bacteriotherapy in a separate study (200, 205). Over the last 5 years, investigators who have used faecal transplantation to treat recurrent cases of CDAD have reported success rates ranging from 86-100% (201, 205-207).The first clinical trial comparing faecal bacteriotherapy against vancomycin was recently conducted by van Nood et al. and an 81% success rate for faecal bacteriotherapy after the first infusion (13/16 cases resolved) was significantly better than the 31% success rate found for vancomycin alone (208).A mix of 33 different bacteria from healthy stool samples was effective in treating CDAD in two patients, in a recent study (209). The etiological agent of the recurring diarrhoea should be confirmed to be *C. difficile* before considering faecal bacteriotherapy as a treatment option.

Faecal bacteriotherapy can lead to alterations in the composition of the gut microbiota. A rise in *Bacteroides*, *Faecalibacterium* and *Roseburia* with a concomitant decrease in *Enterobacteriaeceae* in *C. difficile* patients who are treated with faecal bacteriotherapy is common (206). Hamilton et al. also noted a rise in

Bacteroidetes and *Firmicutes* numbers in 3 patients treated with FMT (207).An increase in *Bacteroidaceae, Porphyromonadaceae* and *Rikenellaceae* families of the *Bacteroidetes* phylum, accompanied by an increase in *Ruminococcaceae* and *Lachnospiraceae* families of the *Firmicutes* phylum following FMT were noted in a study and it has been hypothesised that the presence of such families is associated with gastrointestinal health (207).

In vivo studies involving mouse trials can also be invaluable in terms of providing information and optimizing successful FMT procedures. Six different bacteria were used to cure *C. difficile* R027-infected mice, in a recent *in vivo* trial (210). During CDAD resolution, four out of the six bacterial strains managed to colonize the mice while several other commensals also proliferated, increasing the microbial diversity in doing so. Despite the promising *in vivo* and clinical trial outcomes, there has been a general reluctance in resorting to faecal bacteriotherapy amongst both patients and doctors due to the unattractive nature of the procedure, as well as the extensive screening of donor samples for pathogens that is required prior to transplantation (211).Banking of frozen stool samples which have already been screened for the presence of pathogens, may be a means of expediting the processes involved in performing FMT for CDAD cases.

CONCLUSIONS

The overuse of broad spectrum antibiotics has led to numerous *C. difficile* outbreaks, especially in North America, Canada and Europe over the last two decades. The advent of next-generation sequencing technology in recent years has helped to emphasise the extent of damage caused to the gut microbiota due to broad spectrum

antibiotics. The perturbation of the gut microbiota as a result of antibiotics removes the most potent defence against opportunistic pathogens such as *C. difficile* i.e. the presence of a fully intact gut microbiota. This often leads to a continuous cycle of CDAD and recurrence, as further treatment with broad spectrum antibiotics inhibits the restoration of the commensal gut microbiota, leading to acquisition of CDAD again. Thus, it is clear that there is an urgent need to develop alternative/adjunctive therapeutic options to metronidazole and vancomycin in order to circumvent this ongoing problem of recurrence of disease. Fidaxomicin has already been proved to have promising efficacy as a therapeutic for CDAD. Other antibiotics such as ramoplanin, nitazoxanide, tigecycline and the rifamycin group have shown potent *in vitro* activity and some promising *in vivo* results against *C. difficile* and research is ongoing regarding their clinical efficacy against CDAD. Development of these alternative antibiotics is crucial as the overuse of the current antibiotics metronidazole and vancomycin may lead to development of resistance amongst *C. difficile* targets. Furthermore, there is an inherent risk with the overuse of vancomycin with respect to the spread of vancomycin-resistant enterococci in hospital environments.

The restoration of the commensal gut microbiota using faecal bacteriotherapy has also shown encouraging results in recent years. Faecal bacteriotherapy has tremendous potential in this regard, as it directly tackles the root cause of the problem i.e. dysbiosis caused by broad spectrum antibiotics, resolved by restoring the commensal microbiota via faecal transplantation. Perhaps the most elusive therapeutic option over the years to arrest the cyclic pattern of relapse and recurrence of CDAD, however, has been a narrow spectrum antimicrobial with potent anti-*C. difficile* activity and lack of activity against the commensal gut microbiota. It may be

the case that a narrow spectrum antimicrobial targeted against *C. difficile* and/or restoration of the gut microbiota via FMT may eventually prove to be the most effective treatment regimens for CDAD. Overall, due to the vast number of studies investigating anti-*C. difficile* therapeutic options in recent years, scientists are closer than ever at finally tackling this notorious infection.

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Tables and Figures:

Table 1: Common diagnostic and typing tests to detect *C. difficile* and toxins. Summary of diagnostic methods used to detect *C. difficile*, its toxins or a combination thereof. Common typing methods are also included.

Diagnostic methods	**Detection**
Toxin -culture	Toxigenic *C. difficile*
EIA[a]-toxin A or A/B	Toxin A or A/B
EIA-GDH (Glutamate dehydrogenase)	*C. difficile*
EIA-GDH + EIA toxin A/B	*C. difficile* & toxins
RT-PCR[b] toxin A/B gene	Toxigenic *C. difficile*
Cytotoxin assay	Toxin B (toxin A also has some cytotoxic activity)
NAATs[c]	Toxin genes (usually *tcdB*)
Typing methods	**Principle**
Ribotyping	PCR amplification of spacer regions of 16S and 23S rRNA
Toxinotyping	RFLP-PCR[d] analysis of toxin genes taking into account any mutations within the toxin genes
REA[e]	Use of restriction enzymes that cut bacterial genomic DNA at frequent sites, leading to a large number of DNA fragments
PFGE[f]	Use of a restriction enzyme that cuts genomic DNA at infrequent sites leading to large DNA fragments
MLST[g]	Sequencing of internal fragments of 7 housekeeping genes
MLVA[h]	Based on number of repeat alleles present in the genome following PCR amplification of conserved loci
AFLP[i]	Use of restriction enzymes to cut genomic DNA and subsequent ligation of adaptors to restricted fragments

[a]EIA, enzyme immunoassay; [b]RT-PCR, real-time polymerase chain reaction; [c]NAATs, nucleic acid amplification tests; [d]RFLP-PCR, restriction fragment length polymorphism-polymerase chain reaction; [e]REA, restriction endonuclease analysis; [f]PFGE, pulsed-field gel electrophoresis; [g]MLST, multi-locus sequence typing; [h]MLVA, multilocus variable number tandem repeat analysis; [i]AFLP, amplified fragment length polymorphism.

Table 2: List of *C. difficile* toxinotypes. Toxinotypes of *C. difficile* and toxin production profiles. Adapted from http://www.mf.uni-mb.si/mikro/tox/, Rupnik et al. (96) and Rupnik et al. (117). A^+ and B^+ denote Toxin A and Toxin B production respectively. CDT^+ denotes binary toxin production.

Toxinotypes	Toxins
0	$A^+B^+CDT^-$
I	$A^+B^+CDT^-$
II	$A^+B^+CDT^-$
IIIa	$A^+B^+CDT^+$
IIIb	$A^+B^+CDT^+$
IIIc	$A^+B^+CDT^+$
IV	$A^+B^+CDT^+$
V	$A^+B^+CDT^+$
VI	$A^+B^+CDT^+$
VII	$A^+B^+CDT^+$
VIII	$A^-B^+CDT^-$
IX	$A^+B^+CDT^+$
X	$A^-B^+CDT^+$
XIa	$A^-B^-CDT^+$
XIb	$A^-B^-CDT^+$
XII	$A^+B^+CDT^-$
XIII	$A^+B^+CDT^-$
XIV	$A^+B^+CDT^+$
XV	$A^+B^+CDT^+$
XVI	$A^-B^+CDT^+$
XVII	$A^-B^+CDT^+$
XVIII	$A^+B^+CDT^-$
XIX	$A^+B^+CDT^-$
XX	$A^+B^+CDT^-$
XXI	$A^+B^+CDT^-$
XXII	$A^+B^+CDT^+$
XXIII	$A^+B^+CDT^+$
XXIV	$A^+B^+CDT^+$
XXV	$A^+B^+CDT^+$
XXVI	$A^+B^+CDT^-$
XXVII	$A^+B^+CDT^-$
XXVIII	$A^+B^+CDT^+$
XXIX	$A^+B^+CDT^-$
XXX	$A^-B^+CDT^+$
XXXI	$A^-B^+CDT^+$

Table 3: Summary of minimum inhibitory concentration values (MIC) of anti-*C. difficile* antibiotics and bacteriocins. *In vitro* MICs of alternative antibiotics to metronidazole and vancomycin as well as as *in vitro* MICs of bacteriocins and bioengineered derivatives thereof against *C. difficile* strains as reported in the literature.

Antibiotic	**Description**	**Mode of action**	**MIC range against *C. difficile*(µg/ml)**	**References**
Fidaxomicin	Macrocyclic antibiotic	Inhibition of RNA polymerase	0.008-0.25 (MIC_{90})	190-192
Rifaximin	Rifamycin group	Inhibition of RNA polymerase and transcription	0.0075-0.015 (MIC_{50} and MIC_{90})	157
Rifalazil	Rifamycin group	Inhibition of RNA polymerase and transcription	0.004-0.03	157,193
Nitazoxanide	Nitrothiazole benzamide	Interferes with pyruvate:ferredox in oxidoreductase	0.03-1.0	157,194
Tigecycline	Minocycline derivative	Protein synthesis inhibitor	0.03-0.25 (MIC_{90})	157,195,196
Ramoplanin	Lipoglycodepsipeptide	Inhibition of cell wall synthesis	0.03-0.5 (MIC_{90})	197
Bacteriocin	**Description**	**Mode of action**	**MIC range against *C. difficile*(µg/ml)**	**References**
Thuricin CD	Sactibiotic	Unknown	0.7-2.8	198
Nisin A	Lantibiotic	Inhibition of cell wall synthesis and pore formation	8.38	182
Nisin V (M21V)	Bioengineered derivative of Nisin A	Inhibition of cell wall synthesis and pore formation	4.19	182
Lacticin 3147	Lantibiotic	Inhibition of cell wall synthesis and pore formation	3.6(MIC_{50})	183
Actagardine A	Lantibiotic	Inhibition of cell wall synthesis	1.5-12	184,198
Actagardine V15F	Bioengineered derivative of Actagardine A	Inhibition of cell wall synthesis	2-4	184
LFF571	Thiopeptide derivative	Inhibition of translation by binding elongation factor Tu	0.5-2.0	199

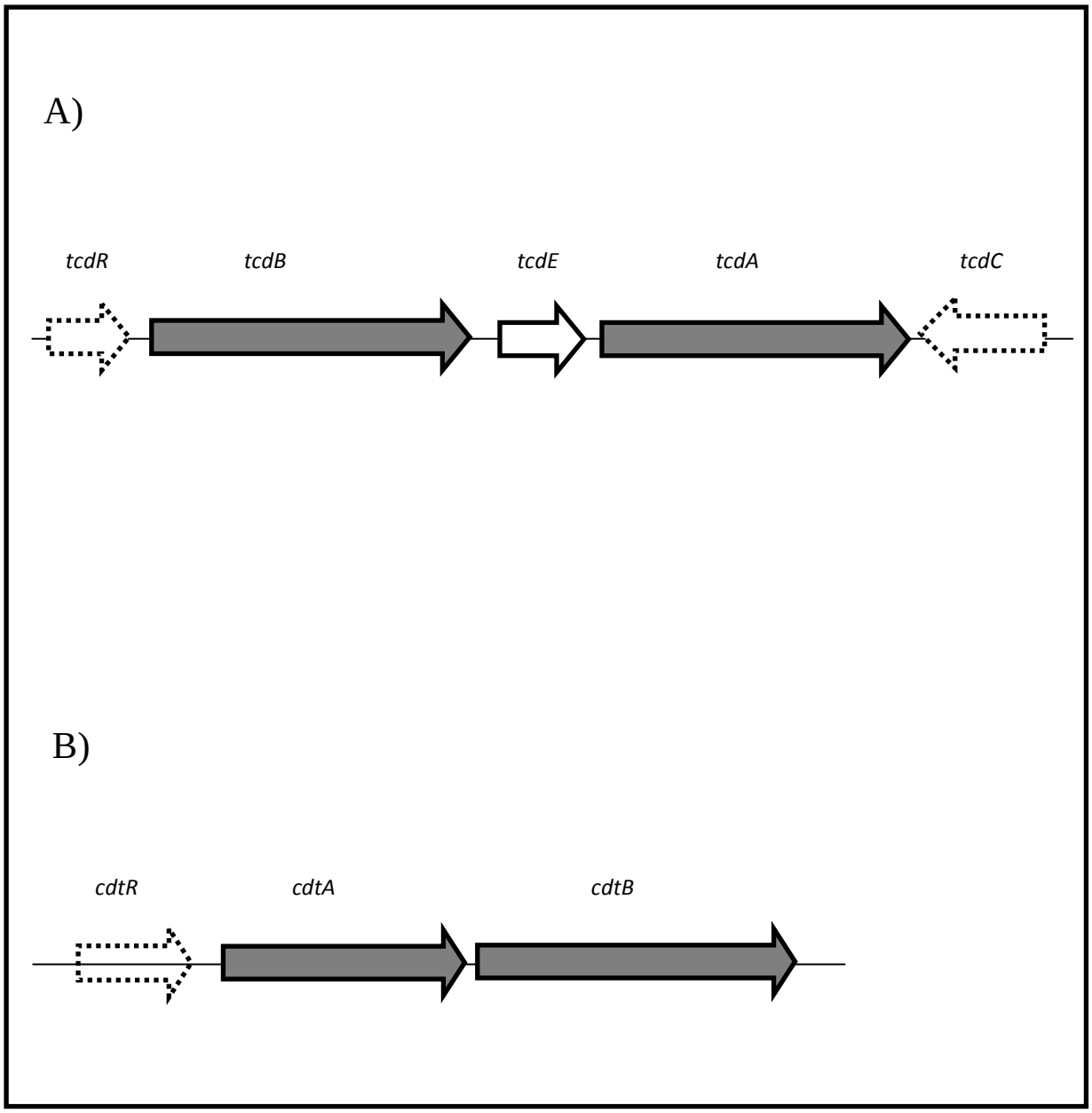
A)
tcdR
tcdB
tcdE
tcdA
tcdC
B)
cdtR
cdtA
cdtB

Figure 1A: *Clostridium difficile* pathogenicity locus (PaLoc).

Schematic organization of the *C. difficile* PaLoc, which is 19.6kb in length. *tcdA* and *tcdB* (shaded in grey) are the two genes encoding the two large *C. difficile* toxins, TcdA and TcdB respectively. *tcdR* (dashed outline) encodes a positive regulator of transcription, whereas *tcdC* (dashed outline) encodes a putative negative regulator/modulatory protein. *tcdE* (shaded in white) encodes a holin protein. Adapted from Dupuy et al. (107) and Carter et al. (108).

Figure 1B: Binary toxin (Cdt) locus.

Schematic organization of the binary toxin locus (6.9kb in length), typically found in outbreak-associated R027 strains. *cdtA* and *cdtB* (shaded in grey) are the two toxin genes. *cdtR* (dashed outline) is the regulatory gene, involved in controlling the amount of binary toxin produced. Adapted from Carter et al. (108).

CHAPTER II

Analysis of the anti-*Clostridium difficile* activity of thuricin CD, vancomycin, metronidazole, ramoplanin and actagardine, both singly and in paired combinations.

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Summary

Clostridium difficile is a nosocomial pathogen and is the etiological agent of *C. difficile*-associated diarrhea (CDAD) and pseudomembranous colitis. The current antibiotics of choice to treat *C. difficile* infection are vancomycin and metronidazole. Other antimicrobial agents with anti-*C. difficile* activity, such as thuricin CD, actagardine and ramoplanin, are at various stages of development. The sensitivity of a collection of clinical *C. difficile* isolates to these antimicrobials, both individually (minimum inhibitory concentrations) and in pairs (fractional inhibitory concentration index), was assessed using the checkerboard microdilution method. The specific activity of thuricin CD against *C. difficile* strains was determined for the first time and molar minimum inhibitory concentration values revealed it to consistently be the most or second most active antimicrobial tested, after ramoplanin. 34 cases of partial synergistic/additive effects were evident out of a total of 117 combinations assessed (29%). The most promising partial synergistic/additive effects were observed when ramoplanin and actagardine were combined. 8 of the 13 strains (61.5%) tested provided FIC indices of between 0.5-1.0 with this combination.

Introduction

The commensal gut microbiota plays a crucial role in the protection of the host against pathogens by means of 'colonization resistance' (1). Perturbations of this gut microbiota due to overuse of broad spectrum antibiotics can have deleterious effects (2, 3, 4, 5). Such disruptions of the commensal gut flora provide opportunities for the overgrowth of the opportunistic pathogen *Clostridium difficile*. *C. difficile* is the etiological agent responsible for approximately 20% of cases of antibiotic-induced diarrhea (AAD) and has been implicated in approximately 90% of cases of pseudomembranous colitis (PMC)(6). The initial treatment regimen for a positive *C. difficile* toxin test and AAD involves the expeditious discontinuation of the offending antibiotic. If the CDAD is mild, 500mg of metronidazole is recommended every 8 hours for 10 days and, in more severe cases, 125mg of vancomycin is administered every 6 hours for 10 days (7). However, treatment failure (14.2% for vancomycin; 22.4% for metronidazole) and recurrence of CDAD (24% for vancomycin; 22.4% for metronidazole) are serious concerns in the clinical setting (8). The danger of the emergence of vancomycin-resistant clostridia and dissemination of vancomycin-resistant enterococci in clinical settings also make it an unattractive treatment choice for mild to moderately severe cases of CDAD (9).

Thus, it is becoming increasingly more important to find other antimicrobials and/or effective antimicrobial combinations against *C. difficile*. Indeed, investigations involving fidaxomicin, rifampicin, fusidic acid and teicoplanin have been reported (10, 11, 12). With respect to combinations of antimicrobials, Bacon et al. (13) reported synergistic activity against 85% of *C. difficile* strains tested when rifampin was combined with bacitracin *in vitro* but Hames et al. (14) found a fractional inhibitory concentration index (FIC) of 1.5-3, i.e. an indicator of indifference, when

metronidazole and vancomycin were combined. Lagrotteria et al. (9) combined metronidazole and rifampin in a randomised clinical trial on patients presenting with early stage CDAD but synergistic effects (15) were not observed. Although tigecycline and rifaximin have shown promising antimicrobial effects against *C. difficile in vitro*, recurring disease has been an issue when administered to patients (16, 17).

In recent years the potency of a number of peptides against *C. difficile* has also been highlighted. Thuricin CD is a sactibiotic (a novel class of post-translationally modified bacteriocins) (18) which exhibits narrow spectrum antimicrobial activity against *C. difficile* isolates (19). As thuricin CD has little impact on other gastrointestinal microorganisms it has been suggested that the recurrence of CDAD, which has been linked with further rounds of antibiotic-mediated microbial perturbations, will be less likely. Actagardine is a member of another group of modified bacteriocins, the lantibiotics. It is active against a range of Gram-positive pathogens such as *C.difficile*, *Staphylococcus aureus*, *Streptococcus pneumonia* and enterococci (20, 21). Finally, ramoplanin is a glycolipodepsipeptide, which displays potent antimicrobial activity against *C. difficile* as well as a number of other targets (22, 23, 24). Ramoplanin, like vancomycin, functions by targeting lipid II in bacterial cell envelopes but, importantly, binds in a manner which is distinct from that of vancomycin (25). Notably, it has been established that ramoplanin remains active against *C. difficile* strains which are relatively resistant to vancomycin or metronidazole (22) and performs well in a hamster model of *C. difficile*-induced colitis (26). Also, a recent clinical study has shown that pseudomembranous colitis (PMC) patients failed to absorb ramoplanin from their GIT, suggesting that its antibacterial activity remained largely localized in the gut (23).

Here we compare the *in vitro* activity of thuricin CD, actagardine, ramoplanin, metronidazole and vancomycin against clinical isolates of *C. difficile* and use checkerboard assays to assess the consequences of using combinations of pairs of these antimicrobials against these targets.

Materials and Methods

Sources of antimicrobials

Thuricin CD was purified as in Rea et al. (18) with some minor modifications. Brain Heart Infusion (BHI) broth was clarified using Amberlite XAD-16 beads (Sigma Aldrich). A single colony of *Bacillus thuringiensis* DPC 6431 was inoculated into 10ml BHI broth and incubated for 16 hours at 37°C with agitation. The overnight culture was sub-cultured twice and 0.1% was sub-cultured into 1L of clarified BHI and incubated at 37°C for 16 hours. The overnight culture was centrifuged at 8000rpm for 15 minutes and the cell free supernatant was passed through 60g of Amberlite beads pre-washed with 1L of distilled water. The beads were washed with 500ml of 40% ethanol and the active fraction eluted in 400ml of 70% isopropanol (IPA) supplemented with 0.1% trifluoroacetic acid (TFA). The cell pellet was treated with 250ml of 70% IPA, 0.1% TFA at 4°C for 4 hours and centrifuged at 8000rpm for 15 minutes. Supernatant S1 (cell extract resuspended in isopropanol) and supernatant S2 (passed through Amberlite beads) were combined and the isopropanol was removed by rotary evaporation (Buchi). The remaining solution was passed through a C-18 column, pre-equilibrated with 60ml of methanol and 60ml of water. The C-18 column was washed with 120ml of 40% ethanol and the active fraction eluted in 100ml of 70% IPA, 0.1% TFA. This preparation was further concentrated by rotary evaporation, prior to separation of the α α and β peptides by HPLC. 4ml aliquots of concentrated preparation were added to a Vydac C8 reverse phase HPLC column (250 x 10mm, 5µ), which was previously equilibrated with 25% acetonitrile and 0.1% TFA. A gradient of 25% acetonitrile (0.1% TFA) to 75% acetonitrile (0.1`% TFA) was developed from 5-40 minutes, using a flow rate of 2.5 ml/min. Vancomycin, metronidazole and ramoplanin were purchased from Sigma-

Aldrich (Vale Road, Arklow, Wicklow, Ireland) and actagardine was kindly provided by Novacta Biosystems Ltd (BioPark Hertfordshire, Broadwater Road, Welwyn Garden City, Hertfordshire, UK).

C. difficile strains

13 *C. difficile* clinical isolates were kindly provided by the Wellcome Trust Sanger Institute, Hinxton, Cambridge, UK. 6 other *C.difficile* strains were obtained from Moorepark Research Centre Culture Collection. All *C. difficile* strains were grown on Fastidious Anaerobic Agar (FAA)(LabM Ltd; Lancashire, UK), supplemented with 7% defibrinated horse blood (TCS Biosciences Ltd; Botolph Claydon, Buckingham, UK) at 37°C in an anaerobic workstation (Davidson & Hardy). The strains were allowed to sporulate on the FAA plates for 48-72 hours. Overnight cultures were grown in Reinforced Clostridial Medium (RCM) broth (Oxoid Ltd; Basingstoke, Hampshire, England) for 16 hours in anaerobic conditions.

Minimum inhibitory concentration (MIC) assays

MIC assays were conducted as in Field et al. (27), with some minor modifications. Thuricin α and β peptides were weighed and resuspended in RCM broth to give a stock concentration of 2µM. Metronidazole, vancomycin, ramoplanin were resuspended in RCM broth as well. Actagardine was resuspended in 50% RCM broth, 50% dimethyl sulphoxide (DMSO). The starting stock concentrations of the antimicrobials were as follows: metronidazole (40µM), vancomycin (20µM), ramoplanin (5µM), actagardine (50µM). Two-fold serial dilutions in RCM were

performed in 96-well microtitre plates in triplicate. Overnight cultures of *C. difficile* were grown in RCM broth for 16 hours at 37°C in an anaerobic workstation. The overnight cultures were sub-cultured at 4% into 10ml RCM broth until mid-log phase (OD_{600} of 0.5) was attained, at which point the culture was diluted 1/5000 and 100µl inoculated into each well in a 96-well plate (inoculum of 5 X 10^5 cfu/ml) to which serially diluted antimicrobial had been added as described above (total volume of 0.2ml). The 96-well plate was incubated for 18 hours in an anaerobic workstation and the MIC determined. The MIC was defined as the lowest concentration of the antimicrobial which completely inhibited the growth of the target *C. difficile* strain after 18 hours.

Broth microdilution checkerboard assay

The consequences of combining pairs of antimicrobials was assessed using the broth microdilution checkerboard assay. These assays were performed as in Orhan et al. (28), with some minor modifications. Briefly, two-fold serial dilutions of the 2 antimicrobial combinations were carried out in the same 96-well plate (whereby antimicrobial A was serially diluted from top to bottom and antimicrobial B was serially diluted from right to left along the 96-well plate) and inoculated with a *C. difficile* strain in a similar manner to an MIC assay. The 96-well plate was incubated anaerobically for 18 hours and any synergistic, additive, indifferent or antagonistic effects were determined. In total, 9 antimicrobial combinations were assessed against 13 *C. difficile* clinical isolates (117 checkerboard assays). The following combinations were tested against each of the 13 *C. difficile* strains: Thuricin CD & metronidazole, thuricin CD & vancomycin, thuricin CD & ramoplanin,

metronidazole & vancomycin, metronidazole & ramoplanin, vancomycin & ramoplanin, metronidazole & actagardine, vancomycin & actagardine, ramoplanin & actagardine. The fractional inhibitory concentration index (FIC) was calculated as follows: $FIC_A + FIC_B$ = (MIC of antimicrobial A in combination/ MIC of antimicrobial A alone) + (MIC of antimicrobial B in combination/MIC of antimicrobial B alone). FIC indices were interpreted as in Bacon et al. (13) and Naghmouchi et al. (29): full synergy ($FIC \leq 0.5$), partial synergy ($0.5 \leq FIC \leq 0.75$), additive ($0.75 \leq FIC \leq 1.0$), indifference ($1.0 \leq FIC \leq 2.0$), antagonistic ($FIC > 2.0$).

Results & Discussion

While the antimicrobial activity of thuricin CD against *C. difficile* has been highlighted previously, the MIC has not previously been determined. When calculated in terms of molar concentrations, the MICs of thuricin CD and ramoplanin were consistently lower than that of metronidazole, vancomycin and actagardine for all 19 strains tested (Table 1). The ramoplanin MIC value was lowest for 13 targets and that for thuricin CD was lowest against 6 strains. These 6 included the 2 hypervirulent *C. difficile* ribotype 027 strains CD196 R027 and R20291 027. Of the other antimicrobials, the MIC of vancomycin was lower than that of metronidazole with respect to 15/19 (79%) of strains assessed and higher than that of metronidazole in only one case. It should be noted that, when expressed in μg/ml, the relative effectiveness of the antimicrobials differed. This was most notable in the case of thuricin CD as a consequence of it consisting of two peptides and thus having a higher combined molecular weight. Actagardine was consistently the least active antimicrobial. However, it is noteworthy that a V15F derivative of actagardine A has recently been found to possess greater anti-*C. difficile* activity (20) and its activity, relative to these other antimicrobials, will merit investigation in the future. In fact, the MIC of V15F has been shown to be 2-4-fold lower than its wild type against 4 *C. difficile* strains tested by Boakes et al. (20). The MIC values we obtained for actagardine against the 19 *C. difficile* strains tested ranged from 1.477 μg/ml to 11.813 μg/ml. Bacon et al. (13) found that the MIC for vancomycin was less than or equal to 1.6 μg/ml and the MIC for metronidazole ranged from 0.8-3.2 μg/ml for their 47 *C. difficile* strains. Others have reported MIC values of between 0.125-2 μg/ml for vancomycin (30) and 0.032-1.0 μg/ml for metronidazole against *C.*

difficile isolates (31). Our MIC values for vancomycin ranged from 0.464-1.856 µg/ml and corresponding metronidazole values from 0.084-0.428 µg/ml. Peláez et al. (22) reported MIC_{50} and MIC_{90} values for ramoplanin against *C. difficile* strains ranging from 0.03-0.5 µg/ml. We determined that MIC values for ramoplanin against the 19 *C. difficile* strains we tested were between 0.088-0.704 µg/ml.

In a previous *in vitro* antimicrobial combination study with *C. difficile*, Bacon et al. (13) observed that bacitracin and rifampin combinations were most effective as synergistic effects were obtained against 29 out of the 34 *C. difficile* strains tested (85%). However, the authors found that metronidazole-rifampin combinations and vancomycin-rifampin combinations were less effective, yielding mainly indifferent effects. Here, out of the 117 checkerboard assay antimicrobial combinations tested, we found 34 cases (29.05%) of partial synergy/additive effects ($0.5 \leq FIC \leq 1.0$). No antagonistic effects were observed (FIC index > 2.0). The other 83 out of the 117 combinations (70.94%) yielded indifferent effects ($1.0 \leq FIC \leq 2.0$). 8 of the antimicrobial combinations were additive against at least one strain, but thuricin CD-metronidazole combinations had indifferent effects against all 13 strains tested. The most promising partial synergistic/additive effects were apparent when actagardine and ramoplanin were combined in that 8 of the 13 (61.5%) strains tested provided FIC indices of between 0.5-1.0. Furthermore, metronidazole and actagardine produced partial synergism/additive effects against 7 of 13 strains (53.4%) tested. Vancomycin and actagardine combinations gave a mixture of partial synergism/additivity (38.46%) and indifferent results. Additive effects were observed for 5/13 strains (38.46%) tested with a combination of ramoplanin and vancomycin. Indeed, ramoplanin-vancomycin, along with ramoplanin-actagardine,

metronidazole-vancomycin and actagardine-vancomycin combinations, yielded additive effects against the most notorious hypervirulent R027 strains. The lowest FIC index obtained was 0.5, an indicator of full synergy, which was observed when ramoplanin was combined with actagardine against *C. difficile* Liv022 R106. FIC indices of 0.508 were also obtained with ramoplanin-actagardine combinations against *C. difficile* CD196 R027, *C. difficile* CD305 R023 strains and vancomycin-actagardine combinations against *C. difficile* CD196 R027. Thuricin CD seemed to be most effective when combined with ramoplanin (4 cases of partial synergism/additivity out of 13 strains) and 2 cases of partial synergism/additivity were evident when thuricin CD was combined with vancomycin. Metronidazole-ramoplanin combinations only yielded 1 case of additive effects. FIC indices of 1.0 were noted for 2 of the 13 strains with metronidazole and vancomycin combinations. In contrast, Hames et al. (14) consistently observed indifferent effects with such a combination against all *C. difficile* strains tested in their study. The absence of antagonism is reassuring as it indicates that there are no deleterious effects when the antimicrobials tested are combined.

The frequency of relapses and recurrences of CDAD following treatment with vancomycin and metronidazole has prompted the search for and development of new antimicrobials to treat the disease. In particular, there are serious concerns about the newly emerged hypervirulent R027 strains which are characterized by their fluoroquinolone resistance and binary toxin production. It is apparent that ramoplanin and thuricin CD, both alone and in combination, exhibit considerable anti-*C. difficile* activity. A number of other partial synergistic/additive effects are also apparent. These antimicrobials, combinations, or other combinations involving

rifamycin or fidaxomicin, may ultimately lead to novel chemotherapeutic treatments for the treatment of CDAD.

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Table1: MIC values of thuricin CD, metronidazole, vancomycin, ramoplanin and actagardine against 19 *C. difficile* strains (including 13 clinical isolates).

C.difficile strain	Thuricin CD **nM/(µg/ml)**	Metronidazole **nM/(µg/ml)**	Vancomycin **nM/(µg/ml)**	Ramoplanin **nM/(µg/ml)**	Actagardine **nM/(µg/ml)**
CF5 R017	250nM **(1.406µg/ml)**	1250nM **(0.214µg/ml)**	1250nM **(1.856µg/ml)**	156nM **(0.352µg/ml)**	6250nM **(11.813µg/ml)**
M68 R017	250nM **(1.406µg/ml)**	1250nM **(0.214µg/ml)**	625nM **(0.928µg/ml)**	156nM **(0.352µg/ml)**	6250nM **(11.813µg/ml)**
630 R012	125nM **(0.703µg/ml)**	1250nM **(0.214µg/ml)**	625nM **(0.928µg/ml)**	156nM **(0.352µg/ml)**	3125nM **(5.907µg/ml)**
CD305 R023	250nM **(1.406µg/ml)**	1250nM **(0.214µg/ml)**	625nM **(0.928µg/ml)**	312nM **(0.704µg/ml)**	6250nM **(11.813µg/ml)**
M120 R078	250nM **(1.406µg/ml)**	1250nM **(0.214µg/ml)**	1250nM **(1.856µg/ml)**	156nM **(0.352µg/ml)**	3125nM **(5.907µg/ml)**
Liv024 R001	500nM **(2.812µg/ml)**	2500nM **(0.428µg/ml)**	625nM **(0.928µg/ml)**	312nM **(0.704µg/ml)**	6250nM **(11.813µg/ml)**
Liv022 R106	250nM **(1.406µg/ml)**	1250nM **(0.214µg/ml)**	1250nM **(1.856µg/ml)**	156nM **(0.352µg/ml)**	6250nM **(11.813µg/ml)**
CD196 R027	250nM **(1.406µg/ml)**	1250nM **(0.214µg/ml)**	625nM **(0.928µg/ml)**	312nM **(0.704µg/ml)**	3125nM **(5.907µg/ml)**
TL178 R002	500nM **(2.812µg/ml)**	2500nM **(0.428µg/ml)**	625nM **(0.928µg/ml)**	156nM **(0.352µg/ml)**	6250nM **(11.813µg/ml)**
TL174 R015	250nM **(1.406µg/ml)**	1250nM **(0.214µg/ml)**	625nM **(0.928µg/ml)**	156nM **(0.352µg/ml)**	3125nM **(5.907µg/ml)**
20291 R027	125nM **(0.703µg/ml)**	1250nM **(0.214µg/ml)**	625nM **(0.928µg/ml)**	156nM **(0.352µg/ml)**	1560nM **(2.953µg/ml)**
TL176 R014	250nM **(1.406µg/ml)**	2500nM **(0.428µg/ml)**	625nM **(0.928µg/ml)**	156nM **(0.352µg/ml)**	6250nM **(11.813µg/ml)**
BI-9 R001	250nM **(1.406µg/ml)**	1250nM **(0.214µg/ml)**	625nM **(0.928µg/ml)**	312nM **(0.704µg/ml)**	6250nM **(11.813µg/ml)**
DPC 6358	125nM **(0.703µg/ml)**	1560nM **(0.267µg/ml)**	312nM **(0.464µg/ml)**	156nM **(0.352µg/ml)**	3125nM **(5.907µg/ml)**
CF9 R001	125nM **(0.703µg/ml)**	980nM **(0.168µg/ml)**	625nM **(0.928µg/ml)**	78nM **(0.176µg/ml)**	3125nM **(5.907µg/ml)**
EM308	125nM **(0.703µg/ml)**	780nM **(0.133µg/ml)**	625nM **(0.928µg/ml)**	78nM **(0.176µg/ml)**	1560M **(2.953µg/ml)**
EM 156	125nM **(0.703µg/ml)**	980nM **(0.168µg/ml)**	625nM **(0.928µg/ml)**	78nM **(0.176µg/ml)**	3125nM **(5.907µg/ml)**
ECM 304	250nM **(1.406µg/ml)**	1560nM **(0.267µg/ml)**	312nM **(0.464µg/ml)**	39nM **(0.088µg/ml)**	781nM **(1.477µg/ml)**
DPC 6508	125nM **(0.703µg/ml)**	490nM **(0.084µg/ml)**	625nM **(0.928µg/ml)**	78nM **(0.176µg/ml)**	3125nM **(5.907µg/ml)**

Table2: FIC values of various combinations of antimicrobials against 13 *C. difficile* clinical isolates.

C.difficile strain	[a]T-M ΣFIC	Activity[b]	T-VAN ΣFIC	Activity	T-R ΣFIC	Activity
CF5 R017	1.01-2.0	I	1.01-2.0	I	1.01-2.0	I
M68 R017	1.01-2.0	I	1.01-2.0	I	1.01-2.0	I
630 R012	1.01-2.0	I	1.01-2.0	I	**0.75**	**PS**
CD305 R023	1.01-2.0	I	1.01-2.0	I	**0.625**	**PS**
M120 R078	1.01-2.0	I	1.01-2.0	I	1.01-2.0	I
Liv024 R001	1.01-2.0	I	**0.625**	**PS**	**1.0**	**Ad**
Liv022 R106	1.01-2.0	I	**1.0**	**Ad**	1.01-2.0	I
CD196 R027	1.01-2.0	I	1.01-2.0	I	1.01-2.0	I
TL178 R002	1.01-2.0	I	1.01-2.0	I	1.01-2.0	I
TL174 R015	1.01-2.0	I	1.01-2.0	I	**0.625**	**PS**
20291R027	1.01-2.0	I	1.01-2.0	I	1.01-2.0	I
TL176 R014	1.01-2.0	I	1.01-2.0	I	1.01-2.0	I
BI-9 R001	1.01-2.0	I	1.01-2.0	I	1.01-2.0	I
C.difficile strain	M-VAN ΣFIC	Activity	M-R ΣFIC	Activity	VAN-R ΣFIC	Activity
CF5 R017	1.01-2.0	I	1.01-2.0	I	1.01-2.0	I
M68 R017	1.01-2.0	I	1.01-2.0	I	1.01-2.0	I
630 R012	1.01-2.0	I	1.01-2.0	I	**1.0**	**Ad**
CD305 R023	1.01-2.0	I	1.01-2.0	I	1.01-2.0	I
M120 R078	1.01-2.0	I	1.01-2.0	I	**1.0**	**Ad**
Liv024 R001	1.01-2.0	I	1.01-2.0	I	**1.0**	**Ad**
Liv022 R106	1.01-2.0	I	**0.75**	**PS**	**0.75**	**PS**
CD196 R027	**1.0**	**Ad**	1.01-2.0	I	1.01-2.0	I
TL178 R002	1.01-2.0	I	1.01-2.0	I	1.01-2.0	I
TL174 R015	1.01-2.0	I	1.01-2.0	I	1.01-2.0	I
20291R027	1.01-2.0	I	1.01-2.0	I	**1.0**	**Ad**
TL176 R014	**1.0**	**Ad**	1.01-2.0	I	1.01-2.0	I
BI-9 R001	1.01-2.0	I	1.01-2.0	I	1.01-2.0	I
C.difficile strain	M-A ΣFIC	Activity	VAN-A ΣFIC	Activity	R-A ΣFIC	Activity
CF5 R017	**1.0**	**Ad**	**1.0**	**Ad**	**0.625**	**PS**
M68 R017	**1.0**	**Ad**	1.01-2.0	I	1.01-2.0	I
630 R012	**1.0**	**Ad**	1.01-2.0	I	1.01-2.0	I
CD305 R023	1.01-2.0	I	1.01-2.0	I	**0.508**	**PS**
M120 R078	**1.0**	**Ad**	**1.0**	**Ad**	**0.75**	**PS**
Liv024 R001	**1.0**	**Ad**	1.01-2.0	I	1.01-2.0	I
Liv022 R106	**1.0**	**Ad**	1.01-2.0	I	**0.5**	**S**
CD196 R027	1.01-2.0	I	**0.508**	**PS**	**0.508**	**PS**
TL178 R002	1.01-2.0	I	**0.75**	**PS**	**0.75**	**PS**
TL174 R015	1.01-2.0	I	**1.0**	**Ad**	**1.0**	**Ad**
20291R027	1.01-2.0	I	1.01-2.0	I	1.01-2.0	I
TL176 R014	**0.75**	**PS**	1.01-2.0	I	**0.75**	**Ad**
BI-9 R001	1.01-2.0	I	1.01-2.0	I	1.01-2.0	I

[a]T, thuricin CD; M, metronidazole; VAN, vancomycin; R, ramoplanin; A, actagardine

[b]S, synergy; PS, partial synergy; Ad, additive; I, indifference.

CHAPTER III

Heterologous expression of thuricin CD immunity genes in *Listeria monocytogenes*.

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Summary

Bacteriocins are ribosomally synthesised peptides that can have a narrow or broad spectrum of antimicrobial activity. Bacteriocin producers typically possess dedicated immunity systems which often consist of an ATP-binding cassette (ABC) transporter system and/or a dedicated immunity protein. Here, we investigate the genes responsible for immunity to thuricin CD, a narrow spectrum two-peptide sactibiotic produced by *Bacillus thuringiensis* DPC6431. Heterologous expression of putative thuricin CD immunity determinants allowed us to identify and investigate the relative importance of the individual genes and gene products that contribute to thuricin CD immunity. We establish that TrnF and TrnG are the individual components of an ABC transporter system that provides immunity to thuricin CD. We also identify a hitherto overlooked open reading frame located upstream of *trnF* predicted to encode a 79 amino acid transmembrane protein. We designate this newly-discovered gene *trnI* and establish that TrnI alone can provide protection against thuricin CD.

Introduction

Thuricin CD is a two-peptide bacteriocin produced by *Bacillus thuringiensis* DPC6431 with potent narrow spectrum activity against *Clostridium difficile* clinical isolates, including hypervirulent strains (1). Both thuricin CD peptides, Trnα and Trnβ, are post-translationally modified resulting in the formation of three sulphur to α-carbon bridges in each peptide (2). As a consequence of this characteristic feature, thuricin CD has been classified as a member of a novel class of bacteriocins designated sactibiotics (**s**ulphur to **a**lpha-**c**arbon) (3). Other sactibiotics include subtilosin A and propionicin F (4, 5). Previous studies with thuricin CD have shown that it is as effective as vancomycin and metronidazole in reducing *C. difficile* numbers in a human distal colon model but, in contrast to the broad-spectrum antibiotics, it did not dramatically impact on other members of the gut microbiota (6). This narrow spectrum activity is advantageous as one of the main causes of *C. difficile*-associated diarrhea (CDAD), and its recurrence, is the 'collateral damage' to gut microbes caused by conventional broad spectrum antibiotics. Although the putative thuricin CD gene cluster has been identified on the basis of bioinformatics analysis, the contribution of the individual genes has not been established (1).

Bacteriocin gene clusters typically contain dedicated immunity genes, required to protect the producer from self-destruction. In the case of bacteriocins from the lantibiotic and other bacteriocin families, these immunity systems typically consist of immunity proteins (designated LanI in lantibiotics) and/or ABC transporter systems (designated LanFE(G) in lantibiotics) (7). These LanI immunity proteins and LanFE(G) ABC transporter systems can act independently or in concert with

each other (7, 8). A non-essential LanH protein is sometimes encoded within lantibiotic gene clusters and functions as an ancillary protein in order to contribute to assembly of a fully functional ABC transporter system (9). ABC transporter systems represent the most common family of proteins found in nature. They typically consist of two highly conserved cytoplasmic ATP binding domains and two hydrophobic integral membrane domains (7).

Perhaps the most extensively studied bacteriocin immunity system is the NisIFEG system associated with the lantibiotic nisin. NisI is a 226 amino acid LanI protein associated with nisin immunity (10). There is little or no homology between the LanI proteins associated with different lantibiotics, which may explain their highly specific nature (10). The NisI protein contains a hydrophobic N-terminal region possessing a consensus lipoprotein sequence. About 50% of NisI is bound to the outside of the cell membrane, whereas the other 50% is secreted from the cell (11). The NisI protein which is not membrane anchored functions by aggregating nisin extracellularly, thus providing immunity to the host cell (12, 13). A study with C-terminally truncated NisI proteins revealed that 21 amino acids located in the C-terminus are essential in providing specific immunity to nisin (14). This was confirmed by the creation of fusion proteins, replacing the C-terminus of SpaI, the LanI protein of the subtilin system, with the 21 C-terminus amino acids from NisI. These fusion proteins were capable of providing immunity to nisin when expressed heterologously in *Lactococcus lactis* (14). While LanI and LanFE(G) are known to function in a co-operative manner in several lantibiotic immunity systems (13, 15), it has been shown that while the C-terminus of NisI is directly involved in binding nisin, it has no role in co-operating with NisFEG (14).

The NisFEG ABC transporter is also involved in nisin immunity (16). NisF contains two ATP-binding sites at the N-terminus. NisE and NisG are primarily hydrophobic proteins and are thought to form the integral membrane domain of the functional ABC transporter system; i.e. the assembled transporter consists of two hydrophobic membrane-bound domains (Nis E and G) and two cytosolic ATP-binding subunits (2 Nis F) (16). Quantification of the cell-bound nisin in *Bacillus subtilis* cells transformed with the *nisFEG* genes indicated that the nisin ABC transporter acts by extruding nisin molecules from the cell membrane and that this function is independent of the NisI protein (10, 17). A variety of *nisF*, *nisE*, *nisG* and *nisI* mutants has revealed that NisI has a larger role in immunity (16), with NisI providing as much as 80% of total immunity (15, 18). It should be noted that there are several varieties of bacteriocin immunity systems but they all generally involve a specialised immunity protein or an ABC-transporter system; e.g. immunity to class IIa, IIb and IId bacteriocins is provided by a specialised immunity protein alone, whereas an ABC-transporter system alone is responsible for immunity to class IIc bacteriocins (19).

Based on previous *in silico* analysis of the thuricin CD cluster, it was hypothesised that TrnF and TrnG are components of an ABC transporter system and thus may be involved either in export of the mature thuricin α and β peptides and/or immunity (1). The putative TrnF and TrnG proteins showed 48% and 28% sequence identity to ATP binding cassette transporter proteins (ABC transporters) respectively (1). Due to the presence of a Walker A consensus motif and a Walker B motif, TrnF was hypothesised to act as the ATP-binding component of an ABC transporter system (1, 20). TrnG was proposed to be an integral membrane component of an ABC transporter system. In light of this, and what is known about ABC transporters, it is

predicted that TrnF and TrnG both dimerise and form a complex with one another at the cell membrane (20, 21). The last gene identified as part of the thuricin CD cluster is *trnE*. *In silico* studies revealed that *trnE* is homologous to the S41-type superfamily of C-terminal processing peptidase genes (1). Given the lack of a signal sequence, *trnE* was predicted to have an intracellular function, perhaps involved in cleavage of the leader sequence from the Trnβ and Trnα pre-peptides and/or a putative immunity function (1).

In this study, we employ heterologous expression as a means of identifying the genes from the thuricin CD cluster that are responsible for providing immunity to the sactibiotic (Fig 1A). We establish that an open reading frame (ORF) located upstream of *trnF*, which we have designated *trnI*, encodes a small dedicated immunity protein and that TrnFG likely constitutes a fully functional ABC transporter system. Both TrnI and TrnFG respectively provide considerable protection against thuricin CD.

Materials and Methods

In silico investigations

In silico investigations of the DNA sequences and amino acid sequences of *trnI* and TrnI respectively, were conducted in order to find homologues using NCBI BLAST (http://blast.ncbi.nlm.nih.gov). A list of homologues of TrnI is included in Table 1. In depth analysis of the TrnI and TrnG proteins was conducted using the software HMMTOP (22), TMHMM (23), SPLIT version 4.0 (24), TMpred (25), SOSUI (26), Phobius (Stockholm Biotechnology Centre) and TopPred(MOBYLE)(27, 28) to predict the membrane topology of the proteins. A number of servers were also used to predict the secondary structure of TrnI including DAS-TMfilter (29), TOPCONS (30), PRED-TMR2 (31), GOR (32), SOPMA (33) and Porter (34).

Growth conditions

E. coli Top10 cells (Invitrogen) were grown in Luria Bertani (LB) broth with vigorous agitation at 37°C. *B. thuringiensis* DPC6431 (1) and *Listeria monocytogenes* 33013 (35) were grown in Brain Heart Infusion (BHI) broth (Oxoid Ltd, Basingstoke, Hampshire, England) with agitation at 37°C. Antibiotics were used at the following concentrations: chloramphenicol 10 µg/ml for both *E. coli* and *L. monocytogenes* 33013 transformants. *Bacillus subtilis* 168, *B. subtilis* JH642, *B. thuringiensis* HD2 and *B. thuringiensis subsp. monterrey* were obtained from the *Bacillus* Genetic Stock Centre (BGSC) and grown in BHI broth and agar at 37°C.

Lactococcus lactis NZ9700 and *L. lactis* subsp. *lactis* DPC3147 were grown at 30°C in M17 broth and agar (Oxoid Ltd, Basingstoke, Hampshire, England) supplemented with 0.5% (wt/vol) glucose (GM17). A list of strains and constructs used in this study are included in Table 2.

General molecular biology techniques

Plasmids were isolated from *E. coli* Top10 cells utilizing the Roche High Pure Plasmid Isolation kit (Roche Diagnostics, Mannheim, Germany), following manufacturer's instructions. Plasmids were isolated from *L. monocytogenes* 33013 in the same manner, following treatment with protoplast buffer (5mM EDTA, 10 mg/ml lysozyme, 0.75M sucrose, 50U/ml mutanolysin, 20mM Tris-HCl pH7.5). Genomic DNA was extracted from *B. thuringiensis* DPC6431 using the Invitrogen PureLink Genomic Extraction kit (Invitrogen, Carlsbad, California, USA). Chemically competent *E. coli* Top10 cells (Invitrogen) were used as intermediate hosts for plasmids pNZ44 (36) and constructs. *L. monocytogenes* 33013 was made electrocompetent as in Monk et al. (37) and electroporations were executed using an Electro cell manipulator (BTX-Harvard apparatus). Polymerase chain reactions were performed using Extensor Hi-Fidelity polymerase (ABgene, Blenheim Road, Epsom, UK) using standard procedures. Plasmids and PCR products were digested with the relevant FastDigest restriction enzymes (Thermo Scientific). PCR products were purified using the GeneJET Gel Extraction Kit (Thermo Scientific). DNA ligations were performed using T4 ligase (Roche Diagnostics, Mannnheim, Germany), according to manufacturer's instructions. Transformants were screened by colony-PCR using 10% Igepal CA-630 (Sigma Aldrich) at 95°C for 12 minutes to access

DNA. Colony PCRs were routinely performed using Taq Polymerase (Bioline). DNA sequencing to ensure the integrity of the constructs was performed by Source BioSciences (Dublin, Ireland). Oligonucleotides used in this study are indicated in Table 3.

Creation of truncated trnI, trnF and trnG

PCRs for cloning were performed using Extensor Hi-Fidelity polymerase (ABgene, Blenheim Road, Epsom, UK). Oligonucleotides used in this study are listed in Table 3. *B. thuringiensis* DPC6431 genomic DNA was used as a template to facilitate creation of N-terminus and C-terminus truncated *trnI*, *trnF* and *trnG* genes. PCR products were digested using Fast-Digest restriction enzymes (Thermo Scientific), cloned downstream of the P44 constitutive promoter in pNZ44 and transformed into chemically competent *E. coli* Top10 cells. Transformants were plated on LB agar, supplemented with 10µg/ml chloramphenicol, screened by colony PCR and sequenced to ensure the integrity of the constructs. Electrocompetent *L. monocytogenes* 33013 cells were made as in Monk et al. (37). The constructs were introduced into electrocompetent *L. monocytogenes* 33013 cells to evaluate the degree of immunity provided by each of the constructs.

Purification of thuricin CD

Thuricin CD β and α peptides were purified as in Rea et al. (1), with some minor modifications. Following the elution of the preparation containing the antimicrobial fraction, the preparation was further concentrated by using a rotary evaporator

(Buchi) and trn β and α peptides were separated by HPLC. Briefly, 4ml aliquots of concentrated preparation were applied to a Proteo Jupiter reverse phase HPLC column (250 x 10mm, 4μ, 90Å), previously equilibrated with 25% acetonitrile containing 0.1% trifluoroacetic acid (TFA). Peptides were eluted from the column using a gradient of 25-75% acetonitrile 0.1% TFA over 35 minutes. The flow rate was 2.5 ml/min and eluent was monitored at 214 nm. Purified actagardine was kindly provided by Novacta Biosciences Ltd.

Minimum inhibitory concentration (MIC) determination

Broth based MIC assays were conducted as in Field et al. (38) with minor alterations. Briefly, *L. monocytogenes* 33013 wild type strains and recombinant constructs were incubated overnight in triplicate in BHI broth at 37°C with agitation. These overnights were sub-cultured into fresh BHI broth at 2% and again incubated at 37°C with agitation until an OD_{600} of approximately 0.5 was attained. At this point, the cultures were diluted 1/5000 and 100μl inoculated into each well in a 96-well microtitre plate (inoculum of approximately 5 X 10^5 cfu/ml) to which two-fold serially diluted antimicrobial had been added (total volume of 0.2ml). The 96-well plate was incubated for 18 hours at 37°C and the MIC determined. The MIC was defined as the lowest concentration of the antimicrobial which completely inhibited the growth of the target strain. Assays were conducted in triplicate.

Kill curve analysis

Time kill studies were conducted as in Field et al. (38) with some minor changes. Overnight cultures containing approximately 10^9 cfu/ml of the relevant indicator strains/constructs were diluted to approximately 10^7 cfu/ml in a final volume of 1ml of BHI broth. A sub-inhibitory concentration of thuricin CD (2.2µM) against the relevant indicator was added in this final volume of 1ml and incubated at 37°C. Aliquots were taken at time points 60 minutes, 180 minutes and 300 minutes and viable cell counts were conducted to enumerate the cell numbers as cfu/ml. Viable counts were performed by diluting aliquots 1/10 in BHI broth and enumeration on BHI agar plates after 24 hours to calculate the numbers killed due to the specific concentration of thuricin CD being tested. All assays were performed in triplicate.

Accession numbers of thuricin CD immunity proteins

The accession numbers for the thuricin CD immunity genes are as follows: TrnI, KJ507827; TrnF, AED99781.1; TrnG, AED99780.1; TrnE, AED99786.1. The newly-discovered immunity protein TrnI (Accession number KJ507827) has been submitted to GenBank (February 2014) and is now part of the thuricin CD gene cluster (Accession number HQ446454.1).

Results

In silico analysis of putative thuricin CD immunity determinants

The thuricin CD gene cluster predicted previously contains seven genes, *trnFGβαCDE* (Fig 1A) (1). It was predicted that *trnF* and *trnG* encode components of an ABC-transporter involved in thuricin CD immunity. *trnE* was predicted to encode a C-terminal processing peptidase belonging to the S41-type superfamily (1). Here, initial *in silico* analysis of the gene cluster has highlighted the presence of two additional ORFs (orf11 and orf12), located upstream of *trnF*. It was speculated that orf11 may encode a transmembrane protein with a putative immunity function based on these *in silico* analyses. Another ORF (orf12) found between orf11 and *trnF* was not predicted to be a gene due to the presence of a Rho-independent transcriptional terminator sequence at the 5' end of orf12. Despite this Rho-independent transcriptional terminator sequence, a ribosome binding site (RBS) was found upstream of *trnF*. A more detailed *in silico* analysis of orf11 (hereafter referred to as *trnI*) and the associated protein TrnI was carried out, using a variety of bioinformatics tools including HMMTOP (22), TMHMM (23), SPLIT version 4.0 (24), TMpred (25), SOSUI (26), Phobius (Stockholm Biotechnology Centre) and TopPred(MOBYLE)(27, 28) as well as the secondary structure prediction servers DAS-TMfilter (29), TOPCONS (30), PRED-TMR2 (31), GOR (32), SOPMA (33) and Porter (34).

All of the above-mentioned topology prediction tools strongly predict that TrnI contains hydrophobic regions, corresponding to two transmembrane domains (TMDs). All predictions indicate that both the N and C-termini of TrnI are located intracellularly, with two transmembrane helices (TMD1 and TMD2) spanning the

cell membrane (Fig 1B). Other than subtle differences, the predicted location of these TMDs is consistent regardless of which *in silico* tool is used. Using the TMpred tool as an example, amino acid residues 26-45 of TMD1 have an outside to inside orientation while amino acid residues 51-72 have an inside to outside orientation, whereas the TMHMM server predicts that residues 28-45 and 50-72 form part of the two transmembrane helices respectively, with residues 46-49 located extracellularly. Tools used to predict the secondary structure of TrnI support the membrane topology prediction tools in that they suggest that TrnI has transmembrane properties. An *in silico* screen for TrnI homologues in other genome sequenced strains and translated draft genome sequences was also conducted using NCBI BLAST (http://blast.ncbi.nlm.nih.gov) using both DNA sequences and amino acid sequences. Using DNA sequences alone, no homologues of *trnI* whatsoever were obtained using BLASTn. However, when using BLASTp and psi-BLAST, six homologues of TrnI were obtained with E-values lower than the default threshold, all in *Bacillus* strains (Table 1). Despite this, no putative conserved domains were found using either BLASTn, BLASTp or psi-BLAST. Furthermore, all homologues of TrnI were hypothetical unknown proteins in *Bacillus* strains, which have yet to be annotated. No homology to known bacteriocin immunity proteins was apparent.

The same topology prediction servers mentioned above were used to predict the topology and orientation of the hydrophobic domains of TrnG, the predicted integral membrane protein of an ABC-transporter system (Fig 1C). All servers predicted the presence of six transmembrane helices. Again, while subtle differences with respect to the predicted location of these TMDs were apparent, there was a broad consensus. TMD1 was predicted to range from amino acid residue 10 to residue 34, TMD2 between residues 38-60, TMD3 between residues 80-107, TMD4 between residues

111-138, TMD5 between residues 142-167 and TMD6 between residues 177-201. All analyses predict that both the N and C-termini of TrnG are found inside the cell. In terms of orientations of the transmembrane, all analyses predicted that TMD 1, 3 and 5 have an inside to outside orientation, whereas TMD 2, 4 and 6 have an outside to inside orientation.

Heterologous expression to identify thuricin CD immunity determinants

To determine the contributions of the products of *trnI*, *trnF*, *trnG* and *trnE* to thuricin CD immunity, the genes were cloned downstream of the constitutive P44 promoter in pNZ44 (to facilitate continuous expression of the cloned genes) and heterologously expressed in the thuricin CD-sensitive strain *L. monocytogenes* 33013 (Table 2). The contribution of the respective genes to thuricin CD immunity was assessed by MIC assays (Table 4) and time-kill studies (Fig 2). While no statistically significant differences ($P > 0.05$) were observed between any of the *L. monocytogenes* immunity constructs and wild type after 1 and 3 hours of incubation in the presence of sub-lethal thuricin CD concentrations (2.2µM), it is apparent after 5 hours that *trnI* provides significant protection against thuricin CD, as can be seen in Figure 2. Expressing *trnI* in *L. monocytogenes* 33013 resulted in an approximately 1.5 log increase in cell numbers after 5 hours of incubation ($P < 0.05$), in contrast with the wild type *L. monocytogenes* 33013 strain, whose numbers decreased after 5 hours (Fig 2). However, *trnE* and *trnG* do not confer the same level of protection as *trnI*, with statistically insignificant ($P > 0.05$) increases in cell numbers even after 5 hours of incubation in the presence of sub-lethal concentrations of thuricin CD (Fig 2). These observations are in agreement with MIC values, whereby the MIC against

thuricin CD was increased to greater than 18µM in the case of strains expressing *trnI*, compared to only a slight increase in *trnG* (3µM) and *trnE* (5µM)-expressing strains, relative to the wild type (2.5µM) (Table 4). *trnF* did not provide any protection (Table 4).

The genes *trnFG*, *trnIF*, *trnIFG* and orf12*trnF* were also cloned into pNZ44. It was apparent that *trnFG*, *trnIFG*, *trnIF* provided significant protection against thuricin CD, with MIC values increasing to >18µM (Table 4). However, orf12*trnF* did not confer any protection. These observations are consistent with viable count analyses, with statistically significant increases ($P < 0.05$) in CFU/ml counts noted for *trnIF*, *trnFG* and *trnIFG*-expressing strains in the presence of 2.2µM thuricin CD relative to the wild type in the presence of thuricin CD after 5 hours of growth (Fig 2). pNZ44*trnIFG* did not provide a greater degree of immunity than pNZ44*trnI* or pNZ44*trnFG* independently, even after 5 hours. The growth of *L. monocytogenes* 33013 wild type in the absence of thuricin CD was comparable to the growth rates of *L. monocytogenes* constructs expressing immunity genes (*trnI*, *trnFG*, *trnIFG* in the presence of thuricin CD) (Fig 2).

Investigations were also carried out to determine if the putative immunity determinants conferred any cross-resistance to other bacteriocins including: thuricin (produced by *B. thuringiensis* HD-2), the sactibiotic subtilosin A, as well as the lantibiotics actagardine, nisin and lacticin 3147, which possess anti-*Listeria* activity. No cross immunity was apparent, regardless of whether MIC or agar based assays were employed (data not shown).

Creation of truncated constructs

In order to gain a better insight into the importance of specific domains within the immunity determinants, we created a number of constructs in which regions of the genes corresponding to the N- or C-terminus of the associated proteins were removed (Table 2). pNZ44*trnI*ΔNT1-13 lacked the first 13 amino acid residues of TrnI and pNZ44*trnI*ΔNT1-30 lacked the amino acid residues 1-30. pNZ44*trnI*ΔCT64-79 and pNZ44*trnI*ΔCT50-79 lacked residues 64-79 and 50-79 of TrnI respectively. pNZ44*trnI*ΔCT64-79 was designed such that the TrnI produced would have a truncated transmembrane region (TMD2) whereas the modified TrnI produced by pNZ44*trnI*ΔCT50-79 would completely be devoid of TMD2. Finally, the TrnI produced by pNZ44*trnI*ΔNT1-13ΔCT64-79 lacked amino acid residues 1-13 and 64-79 of TrnI. The strains producing TrnI proteins devoid of its original N-terminal regions i.e. pNZ44*trnI*ΔNT1-13, pNZ44*trnI*ΔNT1-30 and pNZ44*trnI*ΔNT1-13ΔCT64-79 were no longer resistant to thuricin CD (Table 4). In contrast, strains producing TrnI proteins with C-terminal truncations still provided appreciable levels of protection, with MICs of 18µM noted.

Further constructs were generated from which regions of *trnF* or *trnG* were deleted (Table 2) and subjected to MIC analysis. pNZ44*trnFG*ΔNT1-144 provided only slight protection (MIC 3µM) and pNZ44*trnFG*ΔNT1-144ΔCT93-210 provided no protection (Table 4). The MICs against both pNZ44*trnG*ΔNT1-54 and pNZ44*trnG*ΔCT93-210 were also 2.5µM, identical to the wild type (Table 4). Unsurprisingly, the thuricin CD MIC of pNZ44*trnF*ΔNT 1-144 was also identical to wild type levels of 2.5µM. The construct pNZ44*trnIF*ΔCT195-285 contained the essential ATP-binding Walker A motif at position 33 and Walker B motif at amino

acid position 152 of TrnF. The MICs against pNZ44*trnIF*ΔCT195-285 and pNZ44*trnIFG*ΔCT93-210 were still greater than 18µM (Table 4).

Discussion

We identified the genes responsible for encoding immunity to the two-component sactibiotic thuricin CD. This was facilitated through use of *L. monocytogenes* 33013, also known as *L. monocytogenes* Scott A, a laboratory strain originally associated with an outbreak of listeriosis (39). This strain is sensitive to thuricin CD at concentrations of 2.5µM or higher. Although less sensitive to thuricin CD than the many *C. difficile* strains tested to date, the relative ease with which genes can be sub-cloned and expressed in *L. monocytogenes* made strain 33013 a useful host with respect to these investigations.

Based on previous bioinformatics analysis it was suggested that there were seven genes involved in thuricin CD production, maturation, export and/or immunity (1). *trnF, trnG, trnβ, trnα, trnC, trnD* and *trnE* are each preceded by RBS sequences and their predicted products are homologous, or share features with, other bacteriocin-associated peptides/proteins. Initial *in silico* analysis in this study indicated the potential involvement of two ORFs (orf11 and orf12) located upstream of *trnF* but their roles in thuricin CD production/regulation/immunity were unclear. It was speculated that orf11 could encode a small transmembrane protein. Further extensive *in silico* analysis has provided a more detailed insight into orf11 and we have consequently established that it is actually a small immunity gene (*trnI*), encoding a transmembrane protein (TrnI) which is 79 amino acids in length and 9.09kDa in size. An assessment of TrnI topology using seven topology prediction servers indicates that TrnI contains two transmembrane helices (TMD1 and TMD2). TMHMM and SPLIT4.0 are most reliable (40) and, although there are slight disparities with respect to which amino acid residues are involved in different domains of the protein, both agreed that TrnI possesses two transmembrane regions. Similar TMDs have also

been found within the small immunity proteins of lacticin 3147 as well as the sactibiotic propionicin F, while PepI, the immunity protein associated with Pep5, also contains membrane-associated hydrophobic stretches (41, 42, 43, 44). Such a small immunity protein, most likely associated with the cell membrane due to the presence of the putative TMD sequences, may sequester the thuricin CD peptides or somehow block the insertion of the peptides into the membrane. Immunity proteins generally tend to be very specific and are not easily recognisable in homology searches. Extensive bioinformatics analysis of TrnG (the N-terminal integral membrane component of the ABC transporter system) using seven topology tools was also conducted and it was established that TrnG contains 6 transmembrane helices. As was the case with TrnI, a few minor dissimilarities between the servers were apparent. Nonetheless, the agreement between all seven servers helped to establish that TrnG is likely to be the hydrophobic integral membrane domain of a functional ABC transporter system, TrnFG.

Introduction of pNZ44*trnIFG* into *L. monocytogenes* 33013 conferred the strain with the ability to grow in the presence of thuricin CD, establishing that *trnFG* and/or *trnI* are involved in thuricin CD immunity. The creation and use of pNZ44*trnI* and pNZ44*trnFG* facilitated experiments which established that *trnFG* and *trnI* both provided protection against thuricin CD. The fact that orf11 independently provided protection against thuricin CD confirmed that it is a small immunity gene, henceforth designated *trnI*. Concurrence between biological data and detailed *in silico* analysis in this study lent further credence to the hypothesis that TrnI could function on its own, as it appears to be a small membrane protein, with two TMDs. Unsurprisingly, pNZ44*trnF* and pNZ44orf12*trnF* both failed to provide any immunity to *L. monocytogenes* 33013, since TrnF (the ATP-binding domain of a fully functional

ABC transporter system) is not expected to function without a membrane component. In addition, it proves that orf12 is not involved in thuricin CD immunity. TrnE, a putative peptidase with a probable intracellular function, also confers a minor degree of immunity to the *L. monocytogenes* strain. The exact role of TrnE, an unusual serine-like protease belonging to the S41-superfamily of C-terminal processing peptidases, remains unclear. Generally, such C-terminal processing peptidases are not involved in cleavage of double glycine motifs. TrnE may assume a role in immunity by cleaving the thuricin CD peptides at an alternative site and consequently, restricting the concentration of the bacteriocin in the immediate environment of the producer cell. The precise mechanism remains unclear and merits further investigations.

Cross immunity between bacteriocin producers is a relatively rare occurrence. However, cross immunity between lacticin 481 and nukacin ISK-1 and between epicidin and Pep5 have been reported (8, 45). In this study, we also carried out investigations to determine if *trnIFG* provided cross immunity to thuricin produced by *B. thuringiensis* HD-2 (BGSC), the sactibiotic subtilosin A, as well as the anti-*Listeria* lantibiotics actagardine, nisin and lacticin 3147. All three lantibiotics display potent antimicrobial activity against Gram-positive pathogens, including *L. monocytogenes* and *C. difficile*, similar to thuricin CD (46, 47, 48). However, nisin, lacticin 3147 and actagardine have a broad spectrum of action, in contrast to the highly narrow spectrum of activity of thuricin CD, which is mainly restricted to *C. difficile, L. monocytogenes* and *Bacillus firmus* (1). It was reassuring that thuricin CD immunity determinants did not confer cross immunity to thuricin, subtilosin A, nisin, lacticin 3147 or actagardine as it indicates that thuricin CD and the aforementioned bacteriocins can potentially be used in combination with each other,

with no danger of cross resistance in the rare and unlikely event of target strains acquiring potential immunity gene homologues from the environment. This lack of cross immunity was anticipated, as bacteriocin immunity systems, and in particular dedicated immunity proteins, are highly specific.

The creation and introduction of constructs designed to produce immunity proteins with N and C-terminus truncations provided a better insight into the relative contributions of each of the immunity determinants. The abrogation of the protection provided by TrnI as a consequence of creating derivatives with an N-terminal truncation, lacking either the first 13 amino acids or lacking both the cytoplasmic loop as well as TMD1 highlighted the importance of this region of the protein. As intermediate levels of immunity were provided by C-terminally truncated versions of TrnI, it is apparent that the C-terminus of TrnI and the associated TMD2 are of lesser importance. Due to the predicted membrane location of the immunity protein TrnI, it may be that the receptor for thuricin CD also resides in the membrane. Since immunity proteins generally work by 'target shielding' or bacteriocin interception, it may be the case that TrnI (and in particular the N-terminus associated cytoplasmic loop and TMD1) sequester the thuricin CD peptides. Alternatively, the cytoplasmic loop and TMD1 of TrnI may block a putative membrane receptor for the thuricin CD peptides, consequently preventing the peptides from binding the target site.

Since pNZ44*trnFG*ΔNT1-144 resulted in MIC levels similar to wild type and identical to pNZ44*trnG*, it became clear that the N-terminus of TrnF, containing the essential Walker A motif at amino acid position 33 was important in TrnF functionality. Furthermore, as pNZ44*trnIF*ΔCT195-285 contained a truncated *trnF* gene and still provided immunity, it helped to establish that TrnI is an independent immunity protein, and is not part of a tri-partite ABC-transporter system (consisting

of TrnIFG), as is the case with some lantibiotic gene clusters. One would expect a complete loss of immunity due to a truncated version of the TrnF protein (the ATP-binding component) had TrnI been a component of this ABC-transporter system. In contrast, as pNZ44*trnFG*ΔNT1-144 resulted in a significant loss of immunity, it was definitively established that TrnFG form components of a fully functional bi-partite ABC transporter system. Thus, it is highly likely that the ABC transporter system TrnFG functions by expelling the thuricin CD peptides from the membrane. Lack of the crucial ATP-binding motif of TrnF leads to a loss of functionality of the ABC transporter as the system operates in an energy-dependent manner.

A few other studies have been conducted with respect to immunity to sactibiotics, including propionicin F, subtilosin A and thurincin H (4, 44, 49). The immunity gene for the sactibiotic propionicin F is called *pcfI*. While TrnI contains only two transmembrane helices, PcfI is a small 127 amino acid membrane-bound protein with three transmembrane helices. As is the case with most bacteriocin systems which are highly regulated, the expression of the *pcfI* gene was found to be concomitant with expression of the propionicin F structural gene (44). AlbB is a small 59 amino acid hydrophobic immunity protein involved in providing protection against the sactibiotic subtilosin A (4). While AlbB independently provides immunity, AlbC and AlbD are also required for optimal immunity to subtilosin A. AlbC is an ABC transporter protein, involved in both immunity and export of subtilosin A. Thurincin H is another sactibiotic produced by *B. thuringiensis* (49). The immunity determinants for thurinicin H have not definitively been identified. However, *thnT* encodes a putative ABC transporter protein, with a possible role in immunity while *thnI* is thought to encode a dedicated immunity protein 95 amino acids in length. Interestingly a lack of homologues of *thnI* amongst genome based

sequences was also noted by Lee et al. (49). This reflects the highly specific nature of dedicated immunity proteins. *thnD* and *thnE* are thought to encode the ATP-binding components and permease components of an ABC-transporter system respectively, possibly involved in thurincin H immunity.

In conclusion, this is the first study conducted to investigate immunity to the two-peptide sactibiotic, thuricin CD. We conclude that TrnF and TrnG are the individual components of a dedicated bipartite ABC-transporter system, with TrnE playing a minor role in thuricin CD immunity. It has been experimentally established that TrnI is a dedicated immunity protein. ABC-transporter proteins in bacteriocin systems can be involved in providing both an immunity function as well as an export function. Interestingly, Rea et al. (1) reported that Trnβ and Trnα peptides both possess leader sequences containing a double-glycine motif and cleavage/transport of such leader peptides typically involves a cognate ABC transporter. Indeed, a group of translocator proteins, which contain a supplementary cysteine protease-like N-terminal proteolytic domain, was reported by Havarstein et al. (50). However, TrnF and TrnG do not show any homology to protease enzymes and due to the absence of N-terminus proteolytic sequences, they are highly unlikely to be involved in the cleavage of the thuricin CD leader peptides. Thus, while it is clear that TrnFG are involved in thuricin CD immunity, their role, if any, in the export of Trnα and β peptides requires further study. Regardless of the precise mechanisms involved**,** we have experimentally identified the gene products involved in thuricin CD immunity.

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Tables and Figures

Table 1: Proteins homologous to TrnI using BLASTp and psi-BLAST

(psi-BLAST; http://blast.ncbi.nlm.nih.gov/).

Strain producing homologue	**% identity**	**E-value**	**Accession number of homologue**
Bacillus thuringiensis serovar monterrey BGSC 4AJ1	84	4e-36	WP_000676622.1
Bacillus cereus FRI-35	64	4e-25	YP_006599094.1
Bacillus cereus BAG4X12-1	61	3e-23	WP_000853250.1
Bacillus thuringiensis IBL 200	58	2e-22	WP_003308783.1
Bacillus thuringiensis IBL 4222	56	4e-20	WP_000853266.1
Bacillus cereus BAG1X1-1	54	6e-20	WP_016083289.1

Table 2: List of strains and *L. monocytogenes* 33013 constructs used in this study.

Construct	Description	Source
E. coli Top10 cells	Intermediate cloning host	Invitrogen
L. monocytogenes 33013	Thuricin CD-sensitive strain	Clayton et al. (35)
pNZ44	*E. coli-Listeria* expression	McGrath et al. (36)
B. thuringiensis DPC6431	vector	Rea et al (1)
B. subtilis 168	Thuricin CD producer	BGSC[a]
B. subtilis JH642	Subtilosin A producer	BGSC
B. thuringiensis HD2	Subtilosin A producer	BGSC
B. thuringiensis	Thuricin producer	BGSC
subsp.monterrey	Subtilosin producer	Field et al. (47)
L. lactis NZ9700	Nisin A producer	Draper et al. (43)
L. lactis DPC3147	Lacticin 3147 producer	
pHM16	pNZ44*trnI*	This study
pHM17	pNZ44*trnF*	This study
pHM18	pNZ44*trnG*	This study
pHM19	pNZ44*trnE*	This study
pHM25	pNZ44*trnIFG*	This study
pHM26	pNZ44*trnIF*	This study
pHM27	pNZ44*trnFG*	This study
pHM28	pNZ44orf12*trnF*	This study
pHM29	pNZ44*trnI*ΔNT1-13[b]	This study
pHM30	pNZ44*trnI*ΔNT1-30	This study
pHM31	pNZ44*trnI*ΔNT1-13ΔCT64-79[c]	This study
pHM32	pNZ44*trnI*ΔCT64-79	This study
pHM33	pNZ44*trnI*ΔCT50-79	This study
pHM34	pNZ44*trnFG*ΔNT1-144	This study
pHM35	pNZ44*trnFG*ΔNT1-144 ΔCT93-210	This study
pHM36	pNZ44*trnG*ΔCT93-210	This study
pHM37	pNZ44*trnG*ΔNT1-54	This study
pHM38	pNZ44*trnF*ΔNT1-144	This study
pHM39	pNZ44*trnIFG*ΔCT93-210	This study
pHM40	pNZ44*trnIF*ΔCT195-285	This study

[a]BGSC, *Bacillus* Genetic Stock Centre; [b]ΔNT, N-terminus truncated; [c]ΔCT, C-terminus truncated.

Table 3: List of oligonucleotides used in this study. Restriction sites are underlined and highlighted in bold. [a]ΔNT, N-terminus truncation; [b]ΔCT, C-terminus truncation.

Oligonucleotide	**Sequence 5' to 3'**
pNZ44 MCS For	CTAATGTCACTAACCTGCCCCGTTAG
pNZ44 MCS rev	GGCTATCAATCAAAGCAACACGTG
Pre11 for NcoI	CACTG**CCATGG**AATTTATGCGCTGACTG
Trn rev PstI	GAAC**CTGCAG**TAATATTCAGAAAG
Trn G rev PstI	GAA**CTGCAG**AGATCCTCTTCTTCAAAATG
Trn E for NcoI	CAA**CCATGG**GGTGATTTGTGTGGAATGGAA
trnFforΔNT1-144 NcoI[a]	GCA**CCATGG**GCTATGTCACATAACCCAGAT
trnGrevΔCT1-118 PstI[b]	GAA**CTGCAG**GAGCCAAGGTTACAATCATTTCA
trnFrevΔCT1-91PstI	GAA**CTGCAG**CATCAGTAATATGGGAGGAGA
trnGforΔNT1-54 NcoI	GCA**CCATGG**GGAAAGTCTTGTTACATAGAAG
Orf11rev Pst1	GAA**CTGCAG**CTTCCTAGTATTGGGTGTTAT
Pre12for Nco1	GCA**CCATGG**GCCCAAATAACACCCAATACT
Pre trnF for Nco1	GCA**CCATGG**CTAGATAACATCCTTAGAGTTC
Orf11forΔNT13NcoI	GCA**CCATGG**GGAAATATTCAAAATGCTAAC
Orf11revΔCT16PstI	GAA**CTGCAG**GAATCCTAAGATTATCATTAC
Orf11forΔNT30NcoI	GCA**CCATGG**ACGGGGAGCTGGAGTTACATT
Orf11revΔCT30PstI	GAA**CTGCAG**GGTTGCTTTGTTAAAGCTGTT

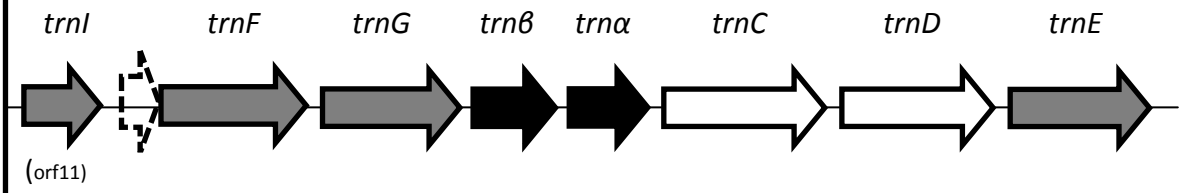
Table 4: MICs of thuricin CD against *L. monocytogenes* 33013 wild type and constructs expressing thuricin CD immunity genes. MICs are indicated in µM with µg/ml values included in parentheses.

Construct	**Thuricin CD MIC**
L. monocytogenes 33013	2.5µM (14.06)
pNZ44	2.5µM (14.06)
pNZ44*trnI*	>18µM(>101.2)
pNZ44*trnF*	2.5µM (14.06)
pNZ44*trnG*	3µM (16.87)
pNZ44*trnE*	5µM (28.12)
pNZ44*trnIFG*	>18µM(>101.2)
pNZ44*trnIF*	>18µM(>101.2)
pNZ44*trnFG*	>18µM(>101.2)
pNZ44orf12*trnF*	2.5µM (14.06)
pNZ44*trnI*ΔNT1-13[a]	2.5µM (14.06)
pNZ44*trnI*ΔNT1-30	2.5µM (14.06)
pNZ44*trnI*ΔNT1-13ΔCT64-79[b]	2.5µM (14.06)
pNZ44*trnI*ΔCT64-79	18µM (101.2)
pNZ44*trnI*ΔCT50-79	18µM (101.2)
pNZ44*trnFG*ΔNT1-144	3µM (16.87)
pNZ44*trnFG*ΔNT1-144 ΔCT93-210	2.5µM (14.06)
pNZ44*trnG*ΔCT93-210	2.5µM (14.06)
pNZ44*trnG*ΔNT1-54	2.5µM (14.06)
pNZ44*trnF*ΔNT1-144	2.5µM (14.06)
pNZ44*trnIFG*ΔCT93-210	>18µM(>101.2)
pNZ44*trnIF*ΔCT195-285	>18µM(>101.2)

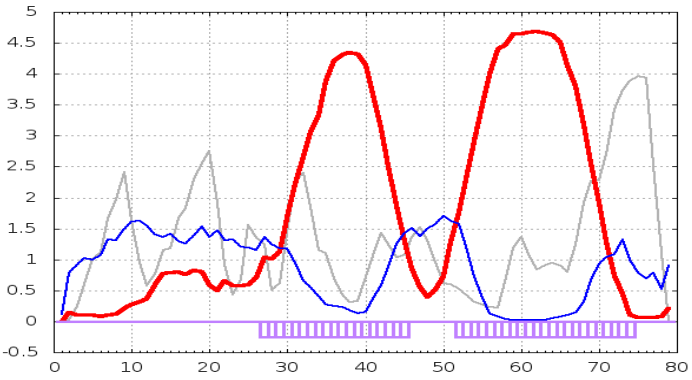
[a]ΔNT, N-terminus truncation; [b]ΔCT, C-terminus truncation. The numbers included after ΔNT and ΔCT indicate the amino acid residues which are excluded from their respective proteins. Values given are identical results from three independent determinations.

Fig 1:

A


trnI
trnF
trnG
trnβ
trnα
trnC
trnD
trnE
(orf11)

B


5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
-0.5
0
10
20
30
40
50
60
70
80

C

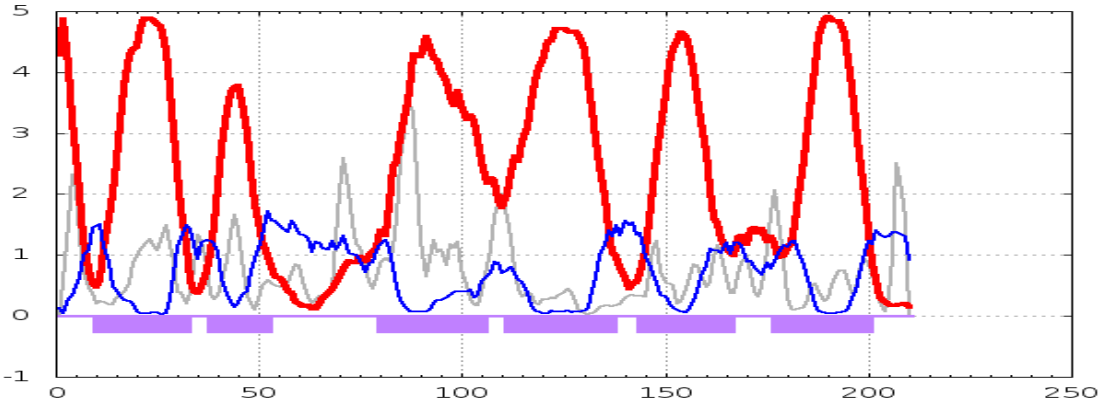

5
4
3
2
1
0
-1
0
50
100
150
200
250

Fig 1:

A) Organisation of the thuricin CD gene cluster. Immunity genes are shaded in gray. *trnI* (formerly orf11) is a small immunity gene encoding a transmembrane immunity protein. *trnFG* encode a fully functional ABC-transporter system. *trnβ* and *trnα* are the two structural genes (shaded in black). *trnC* and *trnD* are the two post-translational modification genes, encoding S-adenosylmethionine proteins (shaded in white). *trnE* encodes a C-terminal processing peptidase with a minor role in thuricin CD immunity (shaded in gray). orf12 (dashed outline), located between *trnI* and *trnF* is unlikely to be a gene. **B) TrnI topology prediction.** Hydrophobicity plot and membrane topology prediction of TrnI based on TMHMM (23). Index: red line; transmembrane helix, blue line; beta preference, gray line; modified hydrophobic moment index, violet boxes; predicted transmembrane helix positions. **C) TrnG topology prediction.** Hydrophobicity plot and membrane topology prediction of TrnG based on TMHMM (23). Index: red line; transmembrane helix, blue line; beta preference, gray line; modified hydrophobic moment index, violet boxes; predicted transmembrane helix positions.

Fig 2:

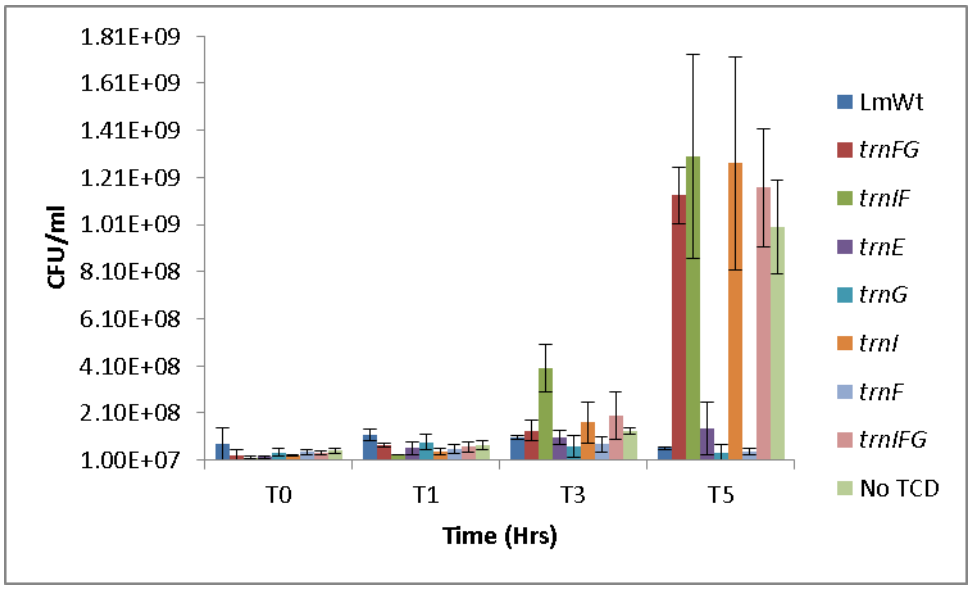


Fig 2: Kill curve analysis. Survival and growth of *L. monocytogenes* 33013 wild type and strains expressing *trnFG*, *trnIF*, *trnE*, *trnG*, *trnI* (formerly orf11), *trnF* and *trnIFG* when challenged with a sub-lethal level (2.2µM) of thuricin CD. The growth of *L. monocytogenes* 33013 in the absence of thuricin CD is also included (No TCD).

CHAPTER IV

Cloning and heterologous expression of the sactibiotic thuricin CD in *Bacillus subtilis*.

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Summary

Thuricin CD is a narrow spectrum two-peptide bacteriocin that displays potent antimicrobial activity against *Clostridium difficile*. Thuricin CD consists of 2 peptides, Trnα and Trnβ, which act in a synergistic manner to kill a variety of *C. difficile* isolates. Each of the peptides is post-translationally modified at amino acid residues 21, 25 and 28. These post-translational modifications result in the formation of three cysteinyl sulphur to α-carbon bridges across the two peptides. Such sulphur to α-carbon bridges are relatively rare in bacteriocins, but have previously been identified in propionicin F and subtilosin A. These bacteriocins, together with thuricin CD, belong to a novel class of bacteriocins referred to as sactibiotics. Here, we report the heterologous production of the sactibiotic thuricin CD in *Bacillus subtilis* 1012. This represents an important step in definitively establishing the composition of the thuricin CD gene cluster. In addition, the importance of *trnCDE* (putative post-translational modification/processing genes) as well as *trnFG* (putative exporters of thuricin CD peptides) is highlighted through the creation of constructs lacking these genes and which are no longer capable of thuricin CD production. To the best of our knowledge, this is the first study describing the heterologous expression of an entire sactibiotic gene cluster.

Introduction

Thuricin CD is a narrow spectrum two-peptide bacteriocin exhibiting potent antimicrobial activity against *Clostridium difficile, Bacillus firmus* and some *Listeria monocytogenes* isolates (1). The producing strain, *Bacillus thuringiensis* DPC6431, was isolated through a screen for anti-*C. difficile* activity from over 30,000 sporeformers isolated from faecal samples. The narrow spectrum of activity of thuricin CD is particularly useful in light of the fact that exposure to broad spectrum antibiotics, and their associated negative impact on the gut microbiota, is a risk factor with respect to the development and recurrence of *C. difficile*-associated diarrhea (CDAD)(1, 2). This narrow spectrum activity of thuricin CD is evident through culture based studies that have demonstrated the absence of activity against many targets, including probiotics from the *Lactobacillus* and *Bifidobacterium* genera (1). Furthermore, while thuricin CD performed as well as the traditional anti-*C. difficile* antibiotics vancomycin and metronidazole in a human distal colon model, it differed from the antibiotics in that it did not induce major fluctuations in the composition of the microbiota (3). Due to this potent, target-specific activity, it would seem that thuricin CD merits consideration as an alternative to metronidazole and vancomycin. Indeed, when the *in vitro* antimicrobial activity of thuricin CD against nineteen *C. difficile* clinical isolates was investigated recently, it emerged that when assessed on the basis of molar concentrations, thuricin CD was consistently more active than metronidazole and vancomycin (4). Furthermore, investigations of the efficacy of thuricin CD in combination with other anti-*C. difficile* antimicrobials revealed partial synergistic/additive effects against 31% of *C. difficile* strains, when combined with ramoplanin (4). Recently, the bioavailability of thuricin CD in the gut has also been studied using murine and porcine models and it

was established that rectal administration of thuricin CD was effective at controlling *C. difficile* numbers (5).

The 3-D structure of thuricin CD has been elucidated and it was shown that both of the thuricin CD peptides, Trnα and Trnβ, contain three sulphur to α-carbon linkages at their 21st, 25th and 28th residues (1, 6). These post-translational modifications are thought to be mediated by radical S-adenosylmethionine (SAM) proteins. Due to these sulphur to α-carbon linkages, which are predicted to be crucial for bioactivity, thuricin CD has been assigned to a novel class of bacteriocins designated sactibiotics (7). Other sactibiotics include subtilosin A and propionicin F (8, 9). Subtilosin A is a cyclic peptide produced by *Bacillus subtilis* that exhibits potent antimicrobial activity against *L. monocytogenes.* The formation of the biologically active form of subtilosin A involves the loss of a seven amino acid leader sequence, N and C-terminal cyclization and subsequent modification of Cys, Thr and Phe residues (9, 10). In the case of subtilosin A, the SAM protein AlbA behaves as an oxidoreductase that catalyzes these modifications (9). Propionicin F is a narrow spectrum sactibiotic produced by *Propionibacterium freudenreichii* that displays antimicrobial activity against other strains of *P. freudenreichii* only (8). Propionicin F is a 43-amino acid bacteriocin which undergoes significant N and C-terminal proteolytic modifications. Indeed, 101 N-terminal and 111 C-terminal residues are removed to yield the mature biologically active 43-amino acid propionicin F peptide. The genetic locus responsible for production of propionicin F consists of a structural gene, *pcfA*, as well as genes thought to encode a SAM protein, a proline peptidase and ABC transporter proteins. A gene located downstream of *pcfA*, designated *pcfB*, encodes a radical SAM protein, the precise role of which has yet to be ascertained (8, 11). Thurincin H is also a sactibiotic produced by another *B. thuringiensis* strain which

displays antimicrobial activity against a variety of Gram-positive bacteria including *Staphylococcus aureus*, *L. monocytogenes* and other *Bacillus* species (7, 12). The mature thurincin H is encoded by the three genes *thnA1*, *thnA2* and *thnA3*, organised in tandem repeats on the *B. thuringiensis* SF361 chromosome. The gene cluster for thurincin H biosynthesis consists of a total of 10 open reading frames (ORFs), three of which are structural genes. Other genes in the thurincin H operon include a bacteriocin processing gene (*thnB*), a transport protein gene (*thnT*), an immunity gene (*thnI*) (12). In addition, a transcription regulator gene (*thnR*), as well as ABC transporter genes are involved while ThnP, a putative leader sequence peptidase, assumes a bacteriocin processing role. The 3-D structure of thurincin H has recently been elucidated and it emerged that thurincin H consists of four sulphur to α-carbon cross links (13).

To date, many different bacteriocins have been expressed in heterologous hosts, including sakacin P (14), nisin (15), divercin V41 (16), enterocin P (17), lacticin 3147 (18), planosporicin (19), labyrinthopeptins (20), leucocin C (21), Ala(0) actagardine (22), pediocin PA-1 (23), enterolysin A (24). However, to date, no sactibiotic gene cluster has been heterologously expressed. Here, we describe the heterologous expression of the sactibiotic thuricin CD in *B. subtilis* 1012, using the *Bacillus* expression vector, pHCMC05. Furthermore, we highlight the importance of the thuricin CD ABC transporter system and post-translational modification/processing genes in thuricin CD production. We show that while the presence of the entire thuricin CD gene cluster results in heterologous production in *B. subtilis*, constructs that lack a subset of genes fail to produce thuricin CD.

Materials and Methods

Strains and growth conditions

B. thuringiensis DPC6431 (1) and *B. subtilis* 1012 (provided by the *Bacillus* Genetic Stock Center, Columbus, USA) were grown in Brain Heart Infusion (BHI) (Oxoid Ltd, Basingstoke, Hampshire, England) agar and broth at 37°C with vigorous agitation. Chemically competent *E. coli* Top10 cells (Invitrogen, Carlsbad, USA) were grown in Luria Bertani (LB) medium supplemented with 1.5% agar. *B. firmus* NRS854 and *B. thuringiensis subsp. huazhongensis* (both provided by the *Bacillus* Genetic Stock Centre, Columbus, USA) were grown on BHI agar and broth at 37°C with vigorous agitation. *C. difficile* indicator strains were kindly provided by the Wellcome Trust Sanger Institute, Hinxton, Cambridge, UK. These *C. difficile* indicator strains were grown on Fastidious Anaerobic Agar (LabM Ltd; Lancashire, UK) supplemented with 7% defibrinated horse blood (TCS Biosciences, Botolph Claydon, Buckingham, UK) for 48 hours in an anaerobic workstation (Davidson & Hardy). Overnight cultures of *C. difficile* strains were grown for 18 hours in Reinforced Clostridium Medium (RCM) broth (Oxoid Ltd; Basingstoke, Hampshire, England) under anaerobic conditions. RCM broth was boiled and allowed to equilibrate in an anaerobic workstation prior to inoculation. *E. coli* Top10 transformants were selected on LB agar plates supplemented with 100µg/ml ampicillin and *B. subtilis* 1012 transformants were selected on BHI agar supplemented with chloramphenicol (7.5µg/ml). A list of strains and constructs used in this study is included in Table 1.

General molecular biology techniques

B. thuringiensis DPC6431 genomic DNA was isolated using the Invitrogen PureLink Genomic Extraction kit (Invitrogen, Carlsbad, California, USA), following manufacturer's instructions. The thuricin CD gene cluster consisting of *trnIFGβαCDE* and the promoter upstream of *trnI* was amplified using the oligonucleotides pre11forBamH1 and trnrevSma1 by PCR using Phusion high-fidelity Polymerase (New England Biolabs, Knowl Piece, Hitchin, UK). The PCR product was digested with BamH1 and Sma1 (Roche Diagnostics, Mannhein, Germany) and ligated into similarly digested pHCMC05 vector (25) using T4 DNA ligase (Roche Diagnostics). The ligation mixture was introduced into chemically competent *E. coli* Top10 cells (Invitrogen) as an intermediate host. *E. coli* transformants were screened by colony-PCR using multiple cloning site and insert primer combinations to identify colonies containing a plasmid with an insert of appropriate size. Such plasmids were extracted from *E. coli* using the Roche High Pure plasmid isolation kit (Roche Diagnostics, Mannhein, Germany) and again checked, through digestion using BamH1 and Sma1, to confirm the presence of an insert of correct size. The recombinant plasmid was then introduced into chemically competent *Bacillus subtilis subsp. subtilis* 1012 cells. pHM2 (*trnβαCDE*) was constructed in the same manner using the trnβforBamH1 and trnrevSma1 oligonucleotide pair while pre11forBamH1 and trnαrev2Sma1 primers were used to construct pHM3 (*trnIFGβα*). The P_{spac} promoter of pHCMC05 was induced with IPTG (Invitrogen). DNA sequencing to ensure the integrity of the constructs was performed by Source BioScience, Dublin, Ireland. A list of oligonucleotides used in this study is included in Table 2.

Development of competence of B. subtilis 1012

B. subtilis 1012 was made naturally competent using the procedure described in Yasbin et al. with some minor modifications (26). Briefly, a 100ml stock of Medium A consisted of the following: 81ml sterile distilled water, 9ml 10x *Bacillus* salts and 10ml of 10x medium A base. Medium A base consisted of yeast extract, casamino acids and glucose. Medium B was made up by adding 100µl of 50mM $CaCl_2.2H_20$ and 100µl of 250mM $MgCl_2.6H_20$ to 10ml of medium A. A few colonies of *B. subtilis* 1012 were inoculated into 20ml of medium A in a 100ml conical flask such that the starting optical density (OD_{600}) was 0.1. This was incubated at 37°C with vigorous agitation. Optical density readings were taken every 30 minutes and T0 was noted at OD_{600} of approximately 0.5. This was the point at which the culture departed logarithmic phase and entered stationary phase. The culture was incubated for a further 90 minutes (T90), at which point, 100µl of the culture was diluted into 900µl of medium B. The diluted culture was again incubated for 90 minutes at 37°C with vigorous agitation. At this point, 250ng of the relevant recombinant pHCMC05 plasmids were added to the competent *B. subtilis* 1012 cells and incubated at 37°C with agitation for a further 30 minutes. *B. subtilis* 1012 transformants were selected on BHI agar plates supplemented with 7.5µg/ml chloramphenicol.

Well diffusion assays

The production of thuricin CD from cell-free supernatants (CFS) of *B. thuringiensis* DPC6431 and *B. subtilis* 1012 constructs was assessed against a range of *C. difficile* and *Bacillus* indicators using the well diffusion assay, as described in Rea et al. (1) with some minor modifications. Briefly, 1ml of overnight cultures of *B.*

thuringiensis DPC6431 and *B. subtilis* 1012 constructs were centrifuged twice in a table-top microcentrifuge at 12,000rpm for 10 minutes. Sensitive *C. difficile* indicator strains were grown overnight for 18 hours in RCM broth in anaerobic conditions. 20ml of molten RCM agar (or BHI agar for *Bacillus* indicators) cooled to 50°C was seeded with 50µl of the overnight culture of the indicator strain (0.25% inoculum). Once the agar solidified, 4.6mm diameter wells were made using a sterile Pasteur pipette and 50µl of the cell free supernatants of *B. thuringiensis* DPC6431 or the *B. subtilis* 1012 constructs were added to the wells. The agar plates were incubated at 37°C for *Bacillus* indicators and in an anaerobic workstation (Davidson & Hardy) at 37°C for *C. difficile* indicators. Diameters of zones of inhibition were measured after overnight growth of the indicators. The pH of BHI broth was adjusted using 2M HCl or 2M NaOH. All assays were performed in triplicate.

Proteinase K sensitivity of antimicrobial-containing cell-free supernatants

The sensitivities of antimicrobial-containing cell-free supernatants (CFS) to proteinase K (Sigma) was assessed using the checkerboard broth microdilution assay, as described by Orhan et al. with some minor modifications (27). Here, instead of using purified antimicrobial peptides and/or antibiotics, we used CFS from overnight cultures of *B. thuringiensis* DPC6431 and *B. subtilis* 1012 (*trnIFGβαCDE*)(pHM149) and proteinase K stocks, diluted in the same manner as in a checkerboard assay. Briefly, the CFS from *B. thuringiensis* DPC6431 and *B. subtilis* (pHM149) was isolated by centrifuging overnight cultures at 12,000rpm for 10 minutes in a table-top microcentrifuge. The cell-free supernatants were then filter-sterilised using 0.45µm filters (Phenomenex). 0.5 mg/ml stock solutions of

proteinase K were made up in BHI broth. 100µl of a proteinase K stock solution was serially diluted from right to left along a microtitre plate and a fixed volume (50µl) of CFS from an overnight culture of *B. subtilis* 1012(pHM149) or *B. thuringiensis* DPC6431 was added to each well. 50µl of a sensitive *Bacillus* indicator containing approximately 5 x 10^5 cells/ml was then introduced into each of the wells such that the final volume was 0.2ml per well. The microtitre plates were incubated at 37°C for 18 hours. The sensitivities of the cell-free supernatants were determined by calculating the minimum concentration of proteinase K which inhibited the antimicrobial activity of thuricin CD present in the supernatants. The relative sensitivities of supernatants from *B. thuringiensis* DPC6431 and *B. subtilis* (pHM149) to proteinase K were compared. The minimum concentration of proteinase K was defined as the lowest concentration of proteinase K at which growth of the indicator strain first appeared (i.e. the least amount of proteinase K required to disrupt the antimicrobial actions of thuricin CD present in the supernatants). This permitted the quantification of active peptides present in the supernatants from both strains, grown under the same conditions. Due to addition of proteinase K, CFS and indicator strains in the same well, all proteinase K concentrations were consequently diluted 1:2, while cell free supernatants were diluted 1:4. The pH of BHI broth was adjusted from pH5-pH9, using 2M HCl and 2M NaOH. All assays were conducted in triplicate.

Results

Heterologous expression of thuricin CD in B. subtilis 1012

The putative thuricin CD gene cluster, consisting of the eight genes *trnIFGβαCDE* (Fig 1), including the native promoter, was cloned downstream of the IPTG-inducible promoter P_{spac} in the *E. coli*/*Bacillus* shuttle vector pHCMC05 and the construct (hereafter designated pHM149) was introduced into *B. subtilis* 1012 to generate *B. subtilis* (pHM149). Well diffusion assays with CFS from overnight cultures of *B. subtilis* (pHM149), using the thuricin CD-sensitive *B. firmus* NRS854 strain as an indicator established that the introduction of pHM149 successfully conferred an antimicrobial-producing phenotype that was not evident in strain *B. subtilis* 1012 (Fig 2). Well diffusion assays were also conducted with 1 mg/ml proteinase K added in wells adjacent to the CFS to ensure that zones of inhibition were due to the thuricin CD peptides (data not shown). Mass spectrometry of *B. subtilis* (pHM149) partially purified preparations was conducted to ensure that the strain was producing thuricin CD (Fig 3).

A range of *C. difficile* indicator strains were used to compare the antimicrobial activity of strain *B. subtilis* (pHM149) relative to the natural *B. thuringiensis* DPC6431 producer. Without IPTG induction, zone sizes produced by *B. subtilis* (pHM149) ranged from 53%-67% of those produced by the natural producer DPC6431. Induction of the P_{spac} promoter within pHM149 with 1mM IPTG resulted in an increase in zone sizes, with values ranging between 71-78%, compared to the natural producer. Induction with higher concentrations of IPTG (5mM) led to a further increase in zone sizes, varying from 76-86%, compared to those produced by DPC6431 (Table 3).

The bioactivity of thuricin CD produced by DPC6431 and *B. subtilis* (pHM149) was also assessed and compared in order to find the optimal pH conditions required for secretion of thuricin CD. Well diffusion assays with supernatants from strains grown under different pH conditions were employed to this end (Table 4). Using the zone sizes obtained with DPC6431 and *B. subtilis* (pHM149) (induced with 1mM IPTG) grown at pH7 as standards, the production levels of thuricin CD at different pH conditions were compared. DPC6431 produced the highest levels of thuricin CD at slightly alkaline pH conditions with zone sizes averaging 119.75% and 126.5% at pH8 and pH9 respectively, relative to zone sizes at pH7 (representing 100%). In contrast, *B. subtilis* (pHM149) did not secrete any thuricin CD into the growth medium when grown at alkaline pH conditions, with a complete lack of zones of inhibition. *B. subtilis* (pHM149) produced the highest levels of thuricin CD at pH7 (Table 4).

Use of proteinase K in order to precisely quantify relative thuricin CD production levels

In order to quantify the precise minimum concentrations of proteinase K required to inhibit the antimicrobial activity of thuricin CD present in supernatants of DPC6431 and *B. subtilis* (pHM149) induced by different concentrations of IPTG, we conducted broth-based checkerboard assays as described by Orhan et al. (27) (Table 5). This assay is the opposite of traditional minimum inhibitory concentration (MIC) assays, whereby the MIC is defined as the lowest concentration of an antimicrobial required to completely inhibit the growth of the target organism after a fixed time point. Here, the minimum concentration of proteinase K required was defined as the

lowest concentration needed to completely inhibit/inactivate the antimicrobial activity of thuricin CD present in the supernatants i.e. the minimum concentration of proteinase K at which growth of the target strain first appeared. Two thuricin CD indicator strains *B. firmus* NRS854 and *B. thuringiensis subsp. huazhongensis* were used for these assays. With respect to *B. firmus* NRS854, 3.906 µg/ml of proteinase K was required to completely inhibit the bioactivity of thuricin CD present in the supernatants of DPC6431. In contrast, only 0.488 µg/ml proteinase K was sufficient to inhibit the bioactivity of supernatants from *B. subtilis* (pHM149) without IPTG induction. 1.953 µg/ml proteinase K was enough to disrupt the antimicrobial actions of thuricin CD in supernatants of *B. subtilis* (pHM149) induced with 1mM IPTG. However, 3.906 µg/ml proteinase K i.e. a quantity identical to that needed inactivate the thuricin CD produced by DPC6431, was required to disrupt the antimicrobial activity of thuricin CD present in the supernatants of *B. subtilis* (pHM149) induced with 5mM IPTG. Against the indicator *B. thuringiensis subsp. huazhongensis*, 1.953 µg/ml proteinase K was the minimum amount required to prevent the antimicrobial activity of thuricin CD present in supernatants of DPC6431. Only 0.244 µg/ml proteinase K was needed to inactivate the antimicrobial component of supernatants from *B. subtilis* (pHM149) without IPTG induction, whereas 0.977 µg/ml proteinase K was needed to completely inhibit the antimicrobial action of supernatants from *B. subtilis* (pHM149) induced with 1mM IPTG. 1.953 µg/ml proteinase K was required to sufficiently inactivate the thuricin CD present in the supernatants from *B. subtilis* (pHM149) induced with 5mM IPTG (Table 5).

Similar proteinase K checkerboard assays were conducted to compare and contrast the relative levels of thuricin CD present in the supernatants of DPC6431 and *B. subtilis* (pHM149) (induced with 1mM IPTG) when grown at different pH

conditions (Table 6). With respect to *B. firmus* NRS854, 3.906 µg/ml proteinase K was the minimum amount required to disrupt the activity of thuricin CD in supernatants of DPC6431, grown at pH5, pH6 and pH7. However, 7.813 µg/ml was needed to completely inhibit the activity of thuricin CD in the supernatants of DPC6431 grown at pH8 and pH9. In contrast, 1.953 µg/ml proteinase K was adequate to disrupt the inhibitory activity of thuricin CD in supernatants of *B. subtilis* (pHM149) (induced with 1mM IPTG) grown at pH5, 6 and 7 against the indicator *B. firmus* NRS854. Identical patterns were apparent against the indicator *B. thuringiensis subsp. huazhongensis*. Against both *B. firmus* NRS854 and *B. thuringiensis subsp. huazhongensis* indicators, the supernatants of *B. subtilis* (pHM149) grown at pH8 and pH9 did not exhibit any antimicrobial activity (Table 6), corroborating with results obtained with well diffusion assays (Table 4).

Deletion of specific genes from the thuricin CD cluster highlights their importance with respect to antimicrobial production

Constructs containing truncated versions of the full thuricin CD gene cluster were made in order to assess the resultant impact on thuricin CD production. PCR products *trnβαCDE* and *trnIFGβα* were cloned into pHCMC05 and designated pHM2 and pHM3 respectively. Although both constructs contained the thuricin CD structural genes (*trnβα*), pHM2 lacked the thuricin CD ABC transporter genes (*trnFG*) as well as the recently-discovered immunity gene *trnI*, whereas pHM3 lacked the putative post-translational modification/processing genes (*trnCDE*). The genes *trnβαCDE* and *trnIFGβα* were again cloned downstream of the IPTG-inducible P_{spac} promoter in pHCMC05 and introduced into electrocompetent *B.*

subtilis 1012 cells to generate *B. subtilis* (pHM2) and *B. subtilis* (pHM3) respectively. While pHM2 lacked the native *B. thuringiensis* promoter located upstream of the entire gene cluster, pHM3 also contained the native promoter, in addition to P_{spac}. The sub-clones *B. subtilis* (pHM2) and *B. subtilis* (pHM3) were tested for bioactivity and both failed to produce thuricin CD (Fig 2).

Discussion

This study describes the process that facilitated the production of thuricin CD in a heterologous host for the first time. The purpose of this 'proof of concept'-type study was to confirm that the putative thuricin CD cluster, previously defined through bioinformatics analysis (1), possessed all of the genes required to facilitate production of the sactibiotic and to assess the importance of a number of genes within the cluster with respect to thuricin CD production. Due to its non-pathogenic and generally regarded as safe (GRAS) status, compounded by its ability to secrete foreign proteins and its quick growth rate, *Bacillus subtilis* was selected as a heterologous host species for expression of thuricin CD (28, 29). Indeed, *B. subtilis* has been used for several years in industry for the production of foods, enzymes, beverages, detergents and other products (28). As *B. subtilis* 1012 is insensitive to thuricin CD and since it belongs to the *Bacillus* genus, and therefore more likely than other genera to express genes from *B. thuringiensis*, it proved to be an ideal host with respect to heterologous expression as well as investigations of export/immunity/modification genes. This is important as the use of constructs containing the thuricin CD structural genes but lacking genes that contribute to immunity could have made these constructs unstable in a thuricin CD-sensitive background.

Although *B. subtilis* is a useful host for production of foreign proteins, the production of extracellular proteases degrading the foreign protein as well as the lack of stable vectors have caused problems in the past. The use of plasmids employing the theta mode of replication has proved successful with respect to overcoming difficulties with stability of vectors. Examples of such plasmids include pAMβ1 and pBS72 (29, 30, 31). More recently, in order to circumvent problems relating to

structural stability of expression vectors to be used in *B. subtilis*, stable vectors based on the shuttle vector pMTLBS72 were constructed by Nguyen and co-workers (25). The use of the expression vector pHCMC05 demonstrating high structural stability (25) was therefore used in this study. The strain *B. subtilis* 1012 has a naturally high ability to secrete foreign proteins. It was recently used by Ilk et al. to express an endotoxin-free S-layer/allergen fusion protein (32).

Bacteriocin gene clusters typically consist of several genes, often organised into operons. These genes include the biosynthetic structural gene(s), immunity gene(s), post-translational modification/processing gene(s), export/translocator gene(s), as well as signal transduction/quorum sensing genes (although these may be located outside the bacteriocin gene cluster) (33, 34, 35, 36, 37). In the majority of cases, the full gene clusters are required for heterologous production of the bacteriocin. However, an exception to the rule includes the sactibiotic propionicin F, which was expressed as a thioredoxin fusion protein in *E. coli* BL21 (DE3) cells, by merely cloning in part of the *pcfA* structural gene (38).

In this study, we first established that the full thuricin CD gene cluster, including the putative *B. thuringiensis* DPC6431 promoter, when cloned downstream of the IPTG-inducible P_{spac} promoter of pHCMC05, successfully conferred a thuricin CD-producing phenotype. Furthermore, the production of thuricin CD in *B. subtilis* 1012 due to the introduction of the full thuricin CD gene cluster (*trnIFGβαCDE*) in pHM149 established that these eight genes are sufficient for thuricin CD production and confirms that open reading frames located upstream and downstream of the thuricin CD gene cluster are highly unlikely to be involved in thuricin CD production, maturation and export. As *B. subtilis* (pHM149) produced thuricin CD without IPTG induction, it was clear that the native thuricin CD promoter was able

to function in the *B. subtilis* host. We then sought to compare and contrast the production levels of thuricin CD by the natural producer (*B. thuringiensis* DPC6431) and *B. subtilis* (pHM149) induced with varying concentrations of IPTG. Unsurprisingly, induction of pHM149 with IPTG resulted in increased levels of thuricin CD production, with 5mM IPTG concentrations resulting in thuricin CD levels comparable to the natural DPC6431 producer.

As assessment of thuricin CD production levels by DPC6431 grown at different pH conditions confirmed that thuricin CD was produced over a wide range of pH conditions as was previously reported (1). In contrast, *B. subtilis* (pHM149) failed to exhibit any bioactivity at alkaline pH conditions. This could perhaps be attributed to the production of extracellular proteases by the *B. subtilis* 1012 host at alkaline pH conditions, which may recognise and degrade foreign peptides. Indeed, studies have described increased efficiencies of enzyme production by *B. subtilis* hosts in slightly alkaline conditions (28, 29). Manabe and co-workers also found increased efficiencies of α-amylase AmyK38 secretion by *B. subtilis* in alkaline conditions (28). In a separate study, the same authors noted a decrease in the secretion of the extracellular enzyme alkaline cellulase Egl-237 in low pH conditions (29). Alternatively, inefficient folding of foreign proteins in the *B. subtilis* 1012 heterologous host under such alkaline conditions may contribute to the lack of thuricin CD activity present in the supernatants.

The use of checkerboard assays in this study, using precise concentrations of proteinase K, permitted the relative quantification of thuricin CD secreted into the growth medium by DPC6431 and *B. subtilis* (pHM149) under different conditions. Similar patterns were observed as in well diffusion assays whereby the levels of production of thuricin CD by *B. subtilis* (pHM149) induced with high concentrations

of IPTG were comparable to DPC6431 production levels. Importantly, both assays clearly demonstrated that a basal level of thuricin CD is still produced by *B. subtilis* (pHM149) without IPTG induction, verifying that the native thuricin CD promoter is indeed functional in the *B. subtilis* 1012 background.

The lack of thuricin CD production by *B. subtilis* (pHM2) and *B. subtilis* (pH3) indicated that the entire set of genes from the thuricin CD gene cluster are most likely required for production, instead of a subset of genes. This is not surprising as bacteriocin production typically requires transport/export, post-translational modification, maturation, regulation, immunity genes, in addition to the biosynthetic structural genes. Prior to this study, *in silico* investigations had indicated that TrnFG are likely components of an ABC transporter system, involved in the export of thuricin CD peptides (1). As *B. subtilis* (pHM2), lacking *trnIFG,* failed to produce thuricin CD, it is likely that TrnFG are involved in export of the thuricin CD peptides out of the cell. TrnI is a small protein and is unlikely to be involved in the export of thuricin CD peptides out of the cell but instead may be involved in sequestering Trnα and Trnβ peptides, serving an immunity function. As *B. subtilis* 1012 is already insensitive to thuricin CD, it is unlikely that the lack of *trnI* has any impact on the viability of the *B. subtilis* host and production of thuricin CD. Therefore, the lack of thuricin CD production by *B. subtilis* (pHM2) is most likely due to a lack of the ABC transporter genes. Such ABC transporters are generally involved in the export of bacteriocins from their respective producer cells (39, 40). Indeed, ABC transporters are amongst the most common proteins found in biological systems (41). They consist of an ATPase domain(s), which is typically highly conserved. This ATPase domain binds and breaks down ATP resulting in the release of energy for downstream processes. Generally, ABC transporter systems are

comprised of 2 hydrophilic ATP binding cassette domains found in the cytoplasm and 2 integral membrane domains which are hydrophobic (42). ABC transporter systems are predominantly associated with the export and import of molecules but can also be associated with other biological processes.

*B. subtili*s (pHM3) lacking *trnCDE*, also failed to produce thuricin CD. This can most likely be attributed to the lack of post-translational modifications due to the absence of the radical S-adenosylmethionine genes (*trnCD)* and consequent lack of formation of the sulphur to α-carbon bridges in both peptides, resulting in a lack of bioactivity. TrnE is a putative C-terminal processing peptidase and plays a minor role in thuricin CD immunity but the precise mechanisms by which it functions remain unclear. As it serves an immunity function, the lack of TrnE is unlikely to have an impact on the ability of *B. subtilis* 1012 to produce thuricin CD. Thus, the lack of thuricin CD production by *B. subtilis* (pHM3) is likely to be due to the absence of *trnC* and *trnD*. Based on previous bioinformatics studies, *trnC* and *trnD* were predicted to encode for post-translational modification genes involved in formation of the characteristic sulphur to α-carbon bridges associated with sactibiotics (1). Similar to TrnC and TrnD, AlbA is the radical S-adenosylmethionine (SAM) enzyme involved in the generation of sulphur to α-carbon crosslinks in the sactibiotic subtilosin A (9, 43). AlbA forms the cross links between cysteines and threonine and between cysteine and two phenylalanines residues before the subtilosin A leader peptide is cleaved off (43). One radical SAM enzyme is predicted to form four sulphur to alpha α-cross links found in the sactibiotic thurincin H (12). Since two radical SAM genes are found in the thuricin CD cluster, it is anticipated that one gene each is responsible for modifying Trnα and Trnβ respectively (1, 6). Such sulphur to α-carbon linkages occur between the cysteinyl sulphur and S21,

T25, T28 residues in Trnα and between the cysteinyl sulphur and T21, A25, Y28 residues in Trnβ.

In conclusion, the production of thuricin CD in a heterologous host has been described for the first time in this study. The thuricin CD production levels by the *B. subtilis* host are comparable to the natural producer, when induced with high concentrations of IPTG. The study also indicates that TrnFG and TrnCD are crucial for bioactivity, as the absence of these proteins led to abrogation of thuricin CD antimicrobial activity.

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Tables and Figures

Table 1: List of strains and constructs used in this study.

Strain/Construct/Plasmid	Source
E. coli Top10 cells	Invitrogen
B. thuringiensis DPC6431	Rea et al 2010
B. subtilis 1012	BGSC[a]
pHCMC05	Nguyen et al 2005
pHM149 (pHCMC05*trnIFGβαCDE*)	This study
pHM2 (pHCMC05Δ*trnIFG*)	This study
pHM3 (pHCMC05Δ*trnCDE*)	This study
B. firmus NRS854	BGSC
B. thuringiensis subsp. huazhongensis	BGSC
C. difficile CF5 017[b]	Sanger Institute
C. difficile M68 017	Sanger Institute
C. difficile 630 012	Sanger Institute
C. difficile BI-9	Sanger Institute
C. difficile CD305 023	Sanger Institute
C. difficile M120 078	Sanger Institute
C. difficile Liv024 001	Sanger Institute
C. difficile TL174 015	Sanger Institute
C. difficile Liv022 106	Sanger Institute
C. difficile CD196 027	Sanger Institute
C. difficile TL178 002	Sanger Institute
C. difficile TL176 014	Sanger Institute

Table 2: Oligonucleotides used in this study. Restriction sites are highlighted in bold and underlined.

Oligonucleotide	**Sequence 5' to 3'**
pHCMC05 MCS for	GGTGTGGCATAATGTGTGGAATTGTG
pHCMC05 MCS rev	TACTGATCAACTGATCCACCTGA
Pre11for BamH1	GTT**GGATCC**CACTGTAATGGAATTTATG
Trn rev Sma1	GA**CCCGGG**TAATATTCAGAAAGTATCT
Trnβ for BamH1	CAG**GGATCC**GAAGAAGAGGATCTTAAA
Trnα rev2 Sma1	CGT**CCCGGG**GTGCTATTCTGTGTAAAA

Table 3: Antimicrobial activity of *B. thuringiensis* DPC6431 versus *B. subtilis* (*trnIFGβαCDE*). Comparison of the bioactivity of *B. thuringiensis* DPC6431[a] and *B. subtilis* 1012 (*trnIFGβαCDE*)[b] induced with different concentrations of IPTG, against *C. difficile* indicator strains, as measured by well diffusion assays. Zone sizes are averages of triplicate experiments. Zone sizes expressed as a percentage (in parentheses) of zone sizes produced by *B. thuringiensis* DPC6431. [c]*C. difficile* strain name, followed by ribotype.

***C. difficile*[c]**	**B.t[a]**	**B.s[b] no IPTG**	**B.s1mM IPTG**	**B.s5mM IPTG**
CF5 017	16.17±0.76	8.83±0.52 **(55)**	11.5±0.87 **(71)**	12.33±0.76 **(76)**
M68 017	16.83±0.76	9.58±0.52 **(57)**	12.5±0.5 **(74)**	13.58±0.52 **(81)**
630 012	17.83±0.58	10.83±0.29 **(61)**	13.25±0.66 **(74)**	14.42±0.63 **(81)**
BI-9	16.0±0.87	8.5±0.5 **(53)**	11.92±0.38 **(75)**	12.33±0.58 **(77)**
CD305 023	16.33±0.29	11.0±0.5 **(67)**	11.83±0.52 **(72)**	13.17±0.58 **(81)**
M120 078	17.33±0.58	10.42±0.63 **(60)**	13.0±0.5 **(75)**	14.17±0.52 **(82)**
Liv24 001	16.5±0.86	11.08±0.63 **(67)**	12.67±0.58 **(77)**	13.67±0.58 **(83)**
TL174 015	16.92±0.52	10.33±0.58 **(61)**	12.75±0.5 **(75)**	14.58±0.52 **(86)**
Liv22 106	17.33±0.58	11.17±0.52 **(64)**	12.75±0.66 **(74)**	14.58±0.72 **(84)**
CD196 027	16.33±0.58	10.92±0.63 **(67)**	12.75±0.43 **(78)**	14.0±0.25 **(86)**
TL178 002	17.33±0.58	10.25±0.43 **(59)**	12.58±0.63 **(73)**	13.83±0.38 **(80)**
TL176 014	17.25±0.66	10.33±0.58 **(60)**	12.5±0.87 **(72)**	14.0±0.25 **(81)**

Table 4: Bioactivity against *C. difficile* indicators at different pH conditions. Zone sizes are averages of triplicate well diffusion assays. Numbers in parentheses indicate zone sizes expressed as percentages of zone sizes obtained with *B. thuringiensis* DPC6431 and *B. subtilis* 1012 (*trnIFGβαCDE*) respectively, grown at pH7. [a]B.t, *Bacillus thuringiensis* DPC6431 grown at different pHs; [b]B.s, *Bacillus subtilis* 1012 (*trnIFGβαCDE*) induced with 1mM IPTG, grown at different pHs; [c]NZ, no zone.[d]*C. difficile* strain name, followed by ribotype.

***C. difficile* strain**[d]	**B.t[a] pH5**	**B.t pH6**	**B.t pH7**	**B.t pH8**	**B.t pH9**
CF5 017	11.58±0.72 **(72)**	11.67±0.58 **(72)**	16.17±0.76	18.33±0.58 **(113)**	19.67±0.58 **(122)**
M68 017	14.0±0.5 **(83)**	13.83±0.76 **(82)**	16.83±0.76	18.58±0.52 **(110)**	20.0±0.87 **(119)**
630 012	13.92±0.29 **(78)**	14.5±0.5 **(81)**	17.83±0.58	19.67±0.58 **(110)**	20.33±0.58 **(114)**
BI-9	14.33±0.58 **(90)**	14.92±0.38 **(93)**	16.0±0.87	19.67±0.58 **(123)**	20.83±0.24 **(130)**
CD305 023	14.67±0.58 **(90)**	15.25±0.25 **(93)**	16.33±0.29	20.33±0.58 **(124)**	21.17±0.24 **(130)**
M120 078	14.58±0.38 **(84)**	15.33±0.58 **(88)**	17.33±0.58	20.67±0.76 **(119)**	21.33±0.42 **(123)**
Liv24 001	15.0±0.5 **(91)**	15.33±0.58 **(93)**	16.5±0.86	20.92±0.38 **(127)**	21.67±0.31 **(131)**
TL174 015	14.67±0.58 **(87)**	15.25±0.66 **(90)**	16.92±0.52	20.5±0.5 **(121)**	22.0±0.41 **(130)**
Liv22 106	14.83±0.58 **(86)**	14.58±0.63 **(84)**	17.33±0.58	21.42±0.52 **(124)**	21.83±0.24 **(126)**
CD196 027	14.17±0.29 **(87)**	15.83±0.29 **(97)**	16.33±0.58	20.33±0.29 **(124)**	22.5±0.41 **(138)**
TL178 002	15.92±0.72 **(92)**	15.67±0.58 **(90)**	17.33±0.58	20.84±1.04 **(120)**	21.83±0.24 **(126)**
TL176 014	14.67±0.58 **(85)**	15.5±0.5 **(90)**	17.25±0.66	21.0±0.43 **(122)**	22.17±0.42 **(129)**
***C. difficile* strain**	**B.s[b] pH5**	**B.s pH6**	**B.s pH7**	**B.s pH8**	**B.s pH9**
CF5 017	10.25±0.43 **(89)**	9.92±0.14 **(86)**	11.5±0.87	NZ[c]	NZ
M68 017	10.25±0.66 **(82)**	9.75±0.25 **(78)**	12.5±0.5	NZ	NZ
630 012	11.67±0.58 **(88)**	11.33±0.58 **(86)**	13.25±0.66	NZ	NZ
BI-9	10.42±0.63 **(87)**	10.25±0.43 **(86)**	11.92±0.38	NZ	NZ
CD305 023	11.0±0.5 **(93)**	10.58±0.72 **(89)**	11.83±0.52	NZ	NZ
M120 078	10.75±0.25 **(83)**	10.92±0.38 **(84)**	13.0±0.5	NZ	NZ
Liv24 001	11.58±0.52 **(91)**	11.25±0.43 **(89)**	12.67±0.58	NZ	NZ
TL174 015	11.17±0.29 **(88)**	10.67±0.29 **(84)**	12.75±0.5	NZ	NZ
Liv22 106	10.58±0.52 **(83)**	10.33±0.58 **(81)**	12.75±0.66	NZ	NZ
CD196 027	10.0±0.25 **(78)**	10.75±0.66 **(84)**	12.75±0.43	NZ	NZ
TL178 002	10.83±0.58 **(86)**	11.17±0.29 **(89)**	12.58±0.63	NZ	NZ
TL176 014	10.67±0.58 **(85)**	11.42±0.38 **(91)**	12.5±0.87	NZ	NZ

Table 5: Proteinase K assays to compare bioactivity of *B. subtilis* (*trnIFGβαCDE*) induced with IPTG. Minimum concentrations of proteinase K required to completely inhibit the antimicrobial activity of thuricin CD present in supernatants of [a]*B. thuringiensis* DPC6431and [b]*B. subtilis* 1012 (*trnIFGβαCDE*) induced with different concentrations of IPTG. Proteinase K concentrations expressed in µg/ml. Supernatants diluted 1:4.

Indicator strain	**B.t[a]**	**B.s[b] no IPTG**	**B.s1mM IPTG**	**B.s5mM IPTG**
B.firmus NRS854	3.906	0.488	1.953	3.906
B. thuringiensis subsp. huazhongensis	1.953	0.244	0.977	1.953

Table 6: Proteinase K assays to compare bioactivity at different pH conditions. Minimum concentrations of proteinase K (expressed in µg/ml) required to inhibit the antimicrobial activity of thuricin CD present in the supernatants of [a]*B. thuringiensis* DPC6431 and [b]*B. subtilis* 1012 (*trnIFGβαCDE*), induced with 1mM IPTG, grown at different pHs. [c] ND; not determined. Supernatants diluted 1:4.

Indicator strain	**B.t[a] pH5**	**B.t pH6**	**B.t pH7**	**B.t pH8**	**B.t pH9**
B.firmus NRS854	3.906	3.906	3.906	7.813	7.813
B. thuringiensis subsp. huazhongensis	1.953	1.953	1.953	3.906	3.906
Indicator strain	**B.s[b] pH5**	**B.s pH6**	**B.s pH7**	**B.s pH8**	**B.s pH9**
B.firmus NRS854	1.953	1.953	1.953	ND[c]	ND
B. thuringiensis subsp. huazhongensis	0.977	0.977	0.977	ND	ND

Fig 1:

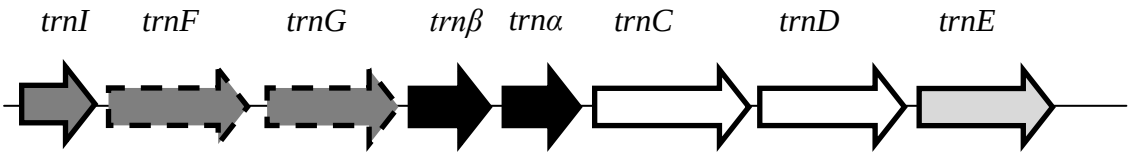

Fig 1: Schematic of the thuricin CD gene cluster. Genetic organisation of the thuricin CD gene cluster containing the eight genes *trnIFGβαCDE*. Genes involved in thuricin CD immunity (*trnIFG*) are shaded in dark grey. *trnFG* genes (dashed outlines) are likely to have dual functionality, involved in both immunity and in export of the thuricin CD peptides. The two structural genes (*trnβ* and *trnα*) are shaded in black. The two post-translational modification genes (*trnCD*), encoding radical S-adenosylmethionine proteins are depicted in white. The last gene in the cluster, *trnE*, shaded in light grey encodes for a peptidase, which may be involved in immunity and/or processing. However, the precise role of *trnE* is unclear.

Fig 2:

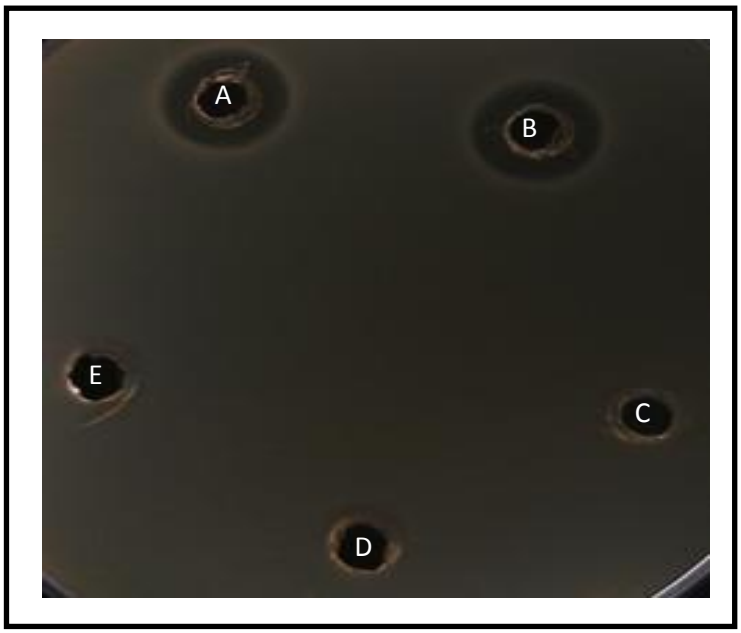

Fig 2. Bioactivity of *B. subtilis* 1012 constructs. Well diffusion assay against *B. firmus* NRS854 using supernatants from A) *B. subtilis* 1012 (*trnIFGβαCDE*) induced with 5mM IPTG; B) *B. thuringiensis* DPC6431; C) *B. subtilis* 1012 wild type; D) *B. subtilis* 1012 (*trnβαCDE*); and E) *B. subtilis* 1012 (*trnIFGβα*).

Fig 3:

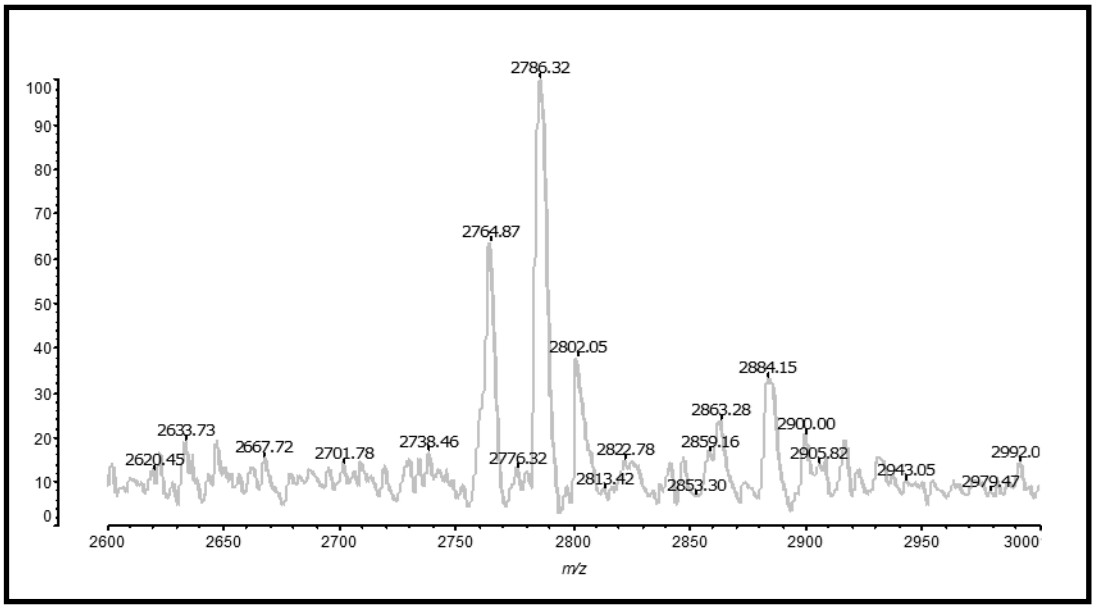

Fig 3. MS spectra of thuricin CD produced by *B. subtilis* 1012 (*trnIFGβαCDE*). Mass spectrometry profile of *B. subtilis* 1012 (*trnIFGβαCDE*) expressing thuricin CD. Predicted mass: Trnα= 2763. Observed mass: Trnα= 2786.32 (addition of Na^+ adduct ion), 2764.87 (addition of H^+ adduct ion) and 2802.05 (addition of K^+ adduct ion). Predicted mass: Trnβ=2861. Observed mass: Trnβ=2884.15 (addition of Na^+ adduct ion) and 2863.28 (addition of H^+ adduct ions).

CHAPTER V

Investigation of low-level resistance to the two-component sactibiotic thuricin CD.

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Manuscript in preparation

Summary

The development of resistance to bacteriocins amongst target microorganisms is a potential concern. We examined whether resistance could occur to the sactibiotic thuricin CD, a bacteriocin with a narrow spectrum of inhibition which includes *Clostridium difficile, Listeria monocytogenes* and *Bacillus firmus*. The purpose of this study was to assess whether resistance to thuricin CD developed amongst different target organisms, investigate the mechanisms of resistance involved and gain an insight into the mode of action of the bacteriocin. Low-level resistance was observed in *L. monocytogenes, B. firmus* and *C. difficile* following repeated exposure to the bacteriocin. Phenotypic assessments of thuricin CD-resistant mutants in this study revealed minor alterations in sensitivities of some mutants to the β-lactam group of antibiotics. It was also established that *L. monocytogenes* mutants exhibited decreased growth rates in the presence of the sugar mannose. To the best of our knowledge, these findings are the first relating to the development of low-level resistance to a bacteriocin from the sactibiotic subclass.

Introduction

Bacteriocins are ribosomally synthesised peptides produced by bacteria, and generally exhibit antimicrobial activity against other (usually closely-related) bacteria (1). Thuricin CD is a bacteriocin produced by *Bacillus thuringiensis* DPC6431 with potent antimicrobial activity against a variety of *Clostridium difficile* virulent isolates as well as some *Bacillus* species and some strains of *Listeria monocytogenes* (2). Thuricin CD consists of two peptides, Trnα and Trnβ, both of which are post-translationally modified at their 21^{st}, 25^{th} and 28^{th} amino acid residues (2, 3). These post-translational modifications involve the formation of three sulphur to α-carbon bridges, which are crucial for thuricin CD antimicrobial activity (2). Recently, a novel nomenclature system has been devised for bacteriocins and thuricin CD has been included in a class of bacteriocins called sactibiotics (4). Other sactibiotics include propionicin F, subtilosin A and its T6I derivative, as well as thurincin H, produced by another strain of *B. thuringiensis* (4, 5, 6, 7). Studies using a human distal colon *ex vivo* model have shown that thuricin CD was as effective as metronidazole and vancomycin at controlling *C. difficile* numbers. However, in contrast to these antibiotics, the bacteriocin had minimal impact on the commensal gut microbiota (8). This is a significantly beneficial trait, as one of the main predisposing factors which leads to recurrent *C. difficile*-associated diarrhea (CDAD) is the extensive collateral damage to the gut microbiota caused by broad-spectrum antibiotics, resulting in a loss of 'colonization resistance' which in turn leads to overgrowth of *C. difficile* (9). Although resistance to metronidazole and vancomycin amongst *C. difficile* has rarely been reported, the overuse of such antibiotics and the development of resistance amongst target *C. difficile* strains remains a realistic

possibility (1, 10, 11, 12). The possibility that *C. difficile* or other targets could become resistant to thuricin CD has not been investigated previously. Indeed, resistance to bacteriocins belonging to the sactibiotic group in general has yet to be reported.

The potency of thuricin CD against *C. difficile* has recently been further highlighted through the determination of minimum inhibitory concentrations (MICs) against nineteen clinical isolates (13). The study revealed that in terms of molar concentrations, thuricin CD was consistently more potent than metronidazole, vancomycin and the lantibiotic actagardine against all nineteen *C. difficile* targets – the MIC of thuricin CD ranged from 0.703 µg/ml-2.812 µg/ml against the nineteen isolates investigated in the study. Furthermore, the efficacy of combinatorial therapy against *C. difficile* was also highlighted in the study, with thuricin CD-ramoplanin and thuricin CD-vancomycin combinations proving most effective (13). Recently, the bioavailability of thuricin CD was also assessed using murine and porcine models of infection (14). Using a murine model, the study revealed that rectal administration of thuricin CD peptides proved to be an effective therapy and resulted in a 1.5 log decrease in *C. difficile* numbers in stool 1 hour after treatment when compared to the control group, whereas a total 3-log decrease was apparent 6 hours post-treatment, relative to the control group (14).

Although resistance to sactibiotics has not been previously investigated, a number of studies investigating the mechanisms of resistance to other bacteriocins have been conducted (15-20). The emergence of intermediate levels of resistance in *Enterococcus faecalis* and *L. monocytogenes* strains to class IIa bacteriocins has previously been reported (16, 17, 20) and has been attributed to attenuated levels of expression of the permease component of the mannose phosphotransferase system

(17, 21, 22, 23). Other studies have reported the development of resistance to the lantibiotic nisin, in a variety of strains including *Streptococcus bovis* (24), *L. monocytogenes* (25-29), *Clostridium botulinum* (27), *Streptococcus thermophilus* (30) which can be attributed to changes in the cell wall, especially in the phospholipid/membrane fatty acid composition (25, 27, 28). Several other factors have been shown to contribute to nisin resistance. A nisin resistance gene, encoding a 35kDa nisin resistance protein (NSR) is responsible for nisin resistance amongst *Lactococcus lactis* strains which do not produce nisin (31). The study showed that NSR is located on the cell membrane and functions as a protease, removing six C-terminal amino acids from nisin. Thus, strains possessing this *nsr* gene are insensitive to nisin. Another study showed that the two component system BraS/BraR is involved in nisin resistance in *Staphylococcus aureus* (32). Kramer et al. conducted DNA-microarray based analysis of nisin-resistant *L. lactis* IL1403 mutants and concluded that the primary mechanisms governing nisin resistance include: i) preventing nisin from actually reaching the membrane, ii) inhibiting nisin insertion into the cell membrane, iii) extruding nisin from the membrane and iv) decreasing the acidity of the extracellular medium causing nisin to bind to the cell wall (33). In another study, Bergholz and co-workers showed that the response regulator LiaR contributes to development of nisin resistance in *L. monocytogenes* in the presence of salt (34). The emergence of spontaneous resistant mutants of *L. lactis* IL1403 with low-level resistance to another lantibiotic, lacticin 3147, was also described in a separate study (35). Regardless of their modes of action, bacteriocins and indeed any antimicrobials which have the potential to be used in the clinic or in food, should be investigated thoroughly with respect to the potential for resistance development amongst target strains. Due to the limited use of bacteriocins in clinical

settings thus far, our knowledge of resistance is largely based on laboratory-based studies. In general, studies investigating attenuated sensitivity to lipid II-targeting bacteriocins have suggested that resistance could be attributed to diminished access to the receptor and/or involves alterations in the bacterial cell envelope (33, 36, 37). Development of resistance to the microcin MccJ25, which has intracellular targets, has been shown to be due to specific mutations in the genes encoding RNA polymerase subunits (38). Mutations in the DNA gyrase-encoding gene has been recognised as a mechanism of resistance development to MccB17 (39).

The objective of this study was to assess the possible development of low, intermediate and high levels of resistance to the novel bacteriocin, thuricin CD. More specifically, we investigated the emergence of thuricin CD resistance in *C. difficile*, *L. monocytogenes* and *B. firmus* strains. It emerged that low-level resistance to thuricin CD was only possible through serial exposure to incrementally increasing concentrations of the bacteriocin. It is notable from these studies that *L. monocytogenes* 33013 mutants exhibiting low-level resistance to thuricin CD also displayed decreased growth rates in the presence of the sugar mannose, with a concomitant increased growth rate in glucose.

Materials and Methods

Bacterial strains and growth conditions

L. monocytogenes 33013 was routinely cultured in Brain Heart Infusion (BHI) (Oxoid Ltd, Basingstoke, Hampshire, England) agar and broth at 37°C with agitation. *B. firmus* NRS854 was also grown on BHI agar and broth at 37°C with vigorous agitation. *C. difficile* strains Liv024 R001, TL174 R015 and CD196 R027 were grown in an anaerobic workstation (Davidson & Hardy) at 37°C on Fastidious Anaerobic Agar (LabM Ltd; Lancashire, UK) supplemented with 7% defibrinated horse blood (TCS Biosciences Ltd; Botolph Claydon, Buckingham, UK) and cycloserine/cefoxitin antibiotics (LabM Ltd). *C. difficile* liquid cultures were routinely grown in Reinforced Clostridium Medium (RCM) (Oxoid Ltd; Basingstoke, Hampshire, England) at 37°C in an anaerobic workstation.

Thuricin CD preparation

Thuricin CD α and β peptides were purified as in Rea et al. with minor modifications (2). Briefly, subsequent to the elution of the preparation containing the antimicrobial fraction, the preparation was subjected to rotary evaporation (Buchi) in order to further concentrate the antimicrobial-containing fraction. The Trnα and Trnβ peptides were then separated by reverse phase-high performance liquid chromatography (RP-HPLC). 4ml volumes of the concentrated preparation were added to a Proteo Jupiter reverse phase-HPLC column (250 x 10mm, 4μ, 90Å). The RP-HPLC column was previously equilibrated with 25% acetonitrile containing 0.1% trifluoroacetic acid (TFA). A gradient of 25-75% acetonitrile 0.1% TFA over

35 minutes was set up, in order to elute the Trnα and Trnβ peptides from the column. A flow rate of 2.5ml/min was used and the eluent was monitored at 214 nm.

Minimum inhibitory concentration determinations

Minimum inhibitory concentration (MIC) assays were conducted as in Field et al., with some minor modifications (40). Briefly, overnight cultures of *L. monocytogenes* 33013, *B. firmus* NRS854, *C. difficile* Liv024 R001, *C. difficile* TL174 R015, *C. difficile* CD196 R027 (and mutants thereof) were grown in the appropriate conditions as mentioned above. The overnight cultures were sub-cultured in BHI broth (for *L. monocytogenes* and *B. firmus*) and RCM broth for *C. difficile* until mid-log phase (OD_{600} of 0.5) was attained, at which point the culture was diluted 1/5000 and 100µl inoculated into each well in a 96-well plate (inoculum of 5 X 10^5 cfu/ml), to which serially diluted antimicrobial had been added (total volume of 0.2ml). The 96-well plates were incubated for 18 hours under appropriate conditions and the MIC determined. The MIC was defined as the lowest concentration of the antimicrobial which completely inhibited the growth of the target strain after 18 hours of incubation. The following antibiotics were purchased from Sigma Aldrich: chloramphenicol, erythromycin, tetracycline, cephradine, ceftazidime hydrate, bacitracin, penicillin G, oxacillin, ampicillin, ramoplanin, metronidazole and vancomycin. Purified actagardine was kindly provided by Novacta Biosystems Ltd. (BioPark Hertfordshire, Broadwater Road, Welwyn Garden City, Hertfordshire, UK). MICs were determined in the same manner as described above for these commercial antibiotics and actagardine and any differences in MICs between wild type and mutant strains were noted. All assays were conducted in triplicate.

Isolation of thuricin CD-resistant mutants

Thuricin CD-resistant mutants were isolated using the same principles as described in Macwana et al. and Severina et al. with some minor alterations (15, 41). Here, instead of using agar-based spot assays and overlay assays, we exposed the thuricin CD indicator strains to precise concentrations of purified thuricin CD by conducting MIC assays as described above and incubating the 96-well plates for prolonged periods of time (24 hours). Any resistant mutants were isolated by plating 20µl of a mixture from a turbid well in a microtitre plate onto BHI agar (for *L. monocytogenes* and *B. firmus*) or Fastidious anaerobic agar, supplemented with 7% defibrinated horse blood and cycloserine/cefoxitin antibiotics (for *C. difficile*). A single colony from each exposure was isolated and further subjected to incrementally increasing concentrations of thuricin CD in 96-well microtitre plates as described above. An isolated colony from each exposure was further subjected to several rounds of serially increasing exposure to thuricin CD and mutants stocked at each stage of resistance development.

Kill curve and growth curve analysis

Time-kill studies and growth curve analysis with *L. monocytogenes* 33013 and mutants thereof were conducted as in Field et al. with some minor modifications (40). Briefly, overnight cultures containing approximately 10^9 cfu/ml of the relevant indicator strains were diluted to 10^7 cfu/ml in a final volume of 1ml of BHI broth. A sub-inhibitory concentration (2.4µM) of thuricin CD against the relevant indicator was added in this final volume of 1ml and incubated at 37°C. Aliquots were taken at time points 60 minutes and 300 minutes and viable cell counts were conducted to

enumerate the cell numbers as cfu/ml. Viable counts were performed by diluting aliquots 1/10 in BHI broth and enumeration on BHI agar plates after 24 hours to calculate the numbers killed due to the specific concentration of thuricin CD being tested. Growth curves experiments were conducted in exactly the same manner as MIC assays as described above with absorbance readings taken hourly at 600nm (OD_{600}) using a Spectromax 340 spectrophotometer (Molecular Devices, Sunnyvale, California) and the sensitivity of *L. monocytogenes* strains to environmental stresses such as salt, pH, lysozyme and cetylpyridinium chloride (Sigma Aldrich) was assessed. The pH of BHI broth was adjusted using 2M HCl and/or 2M NaOH as appropriate. Time-kill studies with *C. difficile* strains were conducted as described by Rea et al. (2). All assays were performed in triplicate.

Genomic DNA extractions

Genomic DNA extractions were performed using the Invitrogen PureLink Genomic Extraction kit (Invitrogen, Carlsbad, California, USA), following manufacturer's instructions. 16S rRNA sequencing was performed using universal 27F and 1492R oligonucleotides to ensure mutants were derivatives of the parental strains. 16S sequencing was performed by Source BioSciences (Dublin, Ireland). Genomic DNA extractions of *C. difficile* wild type strains and their respective thuricin CD-resistant mutants have been sent to the Wellcome Trust Sanger Institute, Cambridge, UK for whole-genome sequencing, in order to gain insights into the mode of action of thuricin CD.

Results

Thuricin CD exhibits highly potent antimicrobial activity against clostridial species, especially *C. difficile*. *C. difficile* is an opportunistic pathogen and *C. difficile* infection primarily occurs due to disruptions of the gut microbiota caused by broad spectrum antibiotics. While thuricin CD has a very narrow spectrum of activity, it also kills some *L. monocytogenes* and *Bacillus* species. In this study we initially attempted to isolate strains of *C. difficile*, *L. monocytogenes* and *B. firmus* with attenuated sensitivity to thuricin CD.

Resistance to thuricin is very low in C. difficile strains

Previous investigations relating to resistance to thuricin CD, using an agar-based approach and direct plating of *C. difficile* to isolate thuricin CD-resistant mutants indicated that the propensity for resistance development was extremely low (frequency of approximately 10^{-9})(Rea et al. unpublished data). For this reason, we attempted to induce resistance to thuricin CD amongst the three *C. difficile* strains (Liv024 R001, TL174 R015 and CD196 R027) by serially exposing each of the strains to incrementally increasing concentrations of thuricin CD. Despite this approach, insensitivity to thuricin CD could only be increased to a maximum of 8-fold after which no growth occurred. Thuricin CD MIC values for *C. difficile* Liv024 R001, *C. difficile* TL174 R015, *C. difficile* CD196 R027 were 2.812 µg/ml, 1.406 µg/ml and 1.406 µg/ml respectively (Table 1). The MICs of thuricin CD against resistant mutants of the above-mentioned strains were 11.248 µg/ml, 8.436µg/ml and 2.812µg/ml respectively. Differences in the MICs of several antibiotics against the wild type *C. difficile* strains and resistant mutants thereof were negligible and merely

differed by two-fold in some cases. Statistically significant differences ($P<0.05$) were apparent between each of *C. difficile* wild type strains and their respective thuricin CD-resistant mutants, after 5 hours of incubation in the presence of sub-lethal 0.225µM (for TL174 R015 and CD196 R027) and 0.45µM (for Liv024 R001) thuricin CD concentrations (Fig 1).

Phenotype of thuricin CD-resistant B. firmus mutants

Similar to *C. difficile*, resistance to thuricin CD was acquired by serially exposing *B. firmus* NRS854 to increasing concentrations of the bacteriocin. The MIC of thuricin CD against wild type *B. firmus* NRS854 was 0.703 µg/ml. Thuricin CD-resistant *B. firmus* NRS854 mutants, designated Bf1-Bf5, had MIC values of 2.812 µg/ml (Bf1, 2 and 3) or 5.624 µg/ml (Bf4 and 5)(Table 2). While no differences in sensitivities of *B. firmus* NRS854 and its thuricin CD-resistant mutants to chloramphenicol (all 2.42µg/ml) and erythromycin (all 18.35µg/ml) were noted, subtle differences in β-lactam antibiotic MIC values were apparent. The MIC values for penicillin G against *B. firmus* mutants were two to four-fold less than those of the wild type *B. firmus* strain (Table 2). Oxacillin and ampicillin MIC values were also two to four-fold less for the *B. firmus* thuricin CD-resistant mutants than the wild type. One mutant, Bf2 displayed a two-fold decrease in sensitivity to cephradine relative to the wild type, with an MIC of 0.436 µg/ml compared to 0.218 µg/ml against *B. firmus* NRS854 wild type. In contrast, two mutants, Bf1 and Bf4, exhibited slight increases in sensitivity to ceftazidime with MICs of 0.683µg/ml and 1.366µg/ml respectively, compared to an MIC of 2.73µg/ml against the wild type. Finally, sensitivity to cetylpyridinium chloride and lysozyme was unaltered (Table 2).

Phenotype of thuricin CD-resistant L. monocytogenes mutants

As was the case with *C. difficile* and *B. firmus* strains, resistance was developed by serially exposing *L. monocytogenes* 33013 to incrementally increasing thuricin CD concentrations in a stepwise manner. The MIC of thuricin CD against wild type *L. monocytogenes* 33013 was 14.06 µg/ml while the *L. monocytogenes* 33013 thuricin CD-resistant mutants designated Lmr1, Lmr2 and Lmr3 had corresponding MICs of 28.12 µg/ml, 56.24 µg/ml and 112.48 µg/ml respectively (Table 3). The resistance/sensitivity of these mutants to other antimicrobials was tested and negligible differences were noted between the wild type and resistant mutants. When grown for 5 hours in the presence of sub-lethal (2.4µM) concentrations of thuricin CD, cfu/ml numbers of the *L. monocytogenes* 33013 wild type merely increased by approximately two-fold of the original starting inoculum. This was significantly less ($P < 0.05$) than the approximately 1.5-log increases in cfu/ml counts for each of Lmr1, Lmr2 and Lmr3 after 5 hours of incubation (Fig 2).

The growth rates of the *L. monocytogenes* strains in BHI broth supplemented with the sugars mannose, galactose or glucose was also compared (Fig 3A, 3B and 3C respectively). In the presence of 5% mannose, Lmr1, Lmr2 and Lmr3 exhibited statistically significant decreased growth rates ($P<0.05$) compared to the wild type (Fig 3A), whereas no differences were apparent in the presence of 5% galactose (Fig 3B). In the presence of 5% glucose, Lmr1, Lmr2 and Lmr3 exhibited statistically significant increased growth rates ($P<0.05$) relative to the wild type (Fig 3C). No differences were apparent when *L. monocytogenes* 33013 wild type and mutants were grown at varying pH conditions of pH5, pH6 and pH7 (Fig 4A, 4B and 4C respectively). Similarly, no differences were apparent between the wild type and mutants under osmotic stress (6.25% sodium chloride), in the presence of lysozyme

or when challenged with the quaternary ammonium compound, cetylpyridinium chloride (Fig 4D, 4E and 4F respectively). The findings of these growth curve analyses indicate that cross resistance amongst thuricin CD-resistant *L. monocytogenes* mutants to environmental stresses such as osmotic stress, pH stress and lysozyme is insignificant.

Discussion

This study is focussed of the emergence of resistant mutants of thuricin CD due to serial exposure to the bacteriocin. A comprehensive assessment of the phenotypic characteristics of such mutants was also conducted, in particular assessing the possibility of cross resistance to other antimicrobials and stressors. The subtle differences in antibiotic susceptibilities across thuricin CD-resistant *L. monocytogenes*, *B. firmus* and *C. difficile* investigated in this study may be due to the different cell wall compositions in these species. Thus, mutations of certain targets as a consequence of thuricin CD resistance development may result in minor variations in antimicrobial sensitivity and differences in phenotypes across species.

The antimicrobial susceptibilities of thuricin CD-resistant *L. monocytogenes*, *B. firmus* and *C. difficile* mutants in terms of MICs were conducted in this study. It was interesting to note that one *L. monocytogenes* 33013 mutant, Lmr3, was two-fold more sensitive to cephradine compared to its wild type parental derivative. Cephradine is a cephalosporin, belonging to the β-lactam class of antibiotics (42). β-lactam antibiotics exert their antimicrobial effects by disrupting the formation of peptidoglycan cross links in bacterial cell walls (43). Penicllin binding proteins (PBPs) are involved in this process of cross linking the peptidoglycan in bacterial cell walls (44). Cephalosporins and other β-lactams inhibit the actions of PBPs such as transpeptidases (45). Altered cephalosporin sensitivity had previously been reported by Guinane et al. with respect to lacticin 3147-resistant variants of *L. lactis* IL1403 (37). In that study, it was found that all eight lacticin 3147-resistant *L. lactis* IL1403 mutants displayed slight increases in sensitivity to cephradine and other cephalosporin antibiotics. A link between altered cephalosporin sensitivity and nisin-resistance in *L. monocytogenes* isolates has also been reported in previous

studies (46, 47). Increased sensitivities of *B. firmus* thuricin CD-resistant mutants to the penicillin group of antibiotics were also apparent in our study. The β-lactam ring of penicillin binds to the PBP DD-transpeptidase, thus inhibiting its ability to catalyse the cross linking of peptidoglycan while at the same time, bacterial enzymes hydrolysing the peptidoglycan cell walls continue working, thus weakening the cell wall and causing cell death (43, 48, 49). The *C. difficile* TL174 R015 thuricin CD-resistant mutant displayed increased sensitivity to the β-lactam ceftazidime. Subtle alterations in sensitivities to the cell wall-targeting antimicrobials ramoplanin, actagardine and vancomycin amongst *C. difficile* mutants indicate that cell wall changes may have taken place as a consequence of thuricin CD resistance development. Interestingly, all 3 thuricin CD-resistant variants of *L. monocytogenes* 33013 displayed two-fold increases in resistance to the macrolide erythromycin, compared to the wild type parental strain. Erythromycin is an antibiotic which possesses bacteriostatic activity by binding to the 50S subunit of the 70S rRNA complex of the bacterial ribosome. It interferes with the transfer of tRNA within the rRNA complex (50). This can potentially be attributed to a mutation in an *erm* gene, resulting in hyper-expression of an erythromycin resistance gene. In a recent study, spontaneous resistant mutants of *S. aureus*, exhibiting resistance to a bacteriocin complex produced by *Bacillus subtilis* LFB112 were isolated (19). Similar to our findings, the resistant *S. aureus* mutants also displayed increased sensitivity to some antimicrobial agents with the exception of chloramphenicol. Interestingly however, changes in membrane fatty acid composition were not thought to be associated with bacteriocin resistance in *S. aureus*, as the compositions of wild type and resistant variants were largely similar in that study (19).

L. monocytogenes 33013 and thuricin CD-resistant Lmr1, Lmr2 and Lmr3 were also subjected to growth curve analysis and time-kill studies in our study. MIC assays are limited by the fact that they provide end-point reads after a fixed time point. In contrast, growth curve assays can illustrate the effects of an antimicrobial or environmental stress such as salt and pH on the viability of the target strain on an ongoing basis over a period of time. All three *L. monocytogenes* thuricin CD-resistant mutants exhibited decreased growth rates in the presence of mannose and increased growth rates in glucose, compared to the parental strain. However, it remains unclear whether this is an indirect adaptive response as a consequence of resistance development or whether the Man-PTS functions as a putative receptor for thuricin CD, and will be the focus of targeted research in the future. Nonetheless, it is tempting to speculate that mutations in the Man-PTS operon of thuricin CD-resistant *L. monocytogenes* mutants may contribute to the apparent differences in metabolism of mannose and glucose. The membrane components IIC and IID of the Man-PTS act as receptors for bacteriocins such as the class IIa pediocin-like bacteriocins, as well as the class IIc bacteriocin lactococcin A (16, 51, 52, 53). While lactococcin A merely targets the Man-PTS from *Lactococcus* strains, the Man-PTS from several genera including *Listeria*, *Enterococcus* and *Lactobacillus* is targeted by class IIa bacteriocins (54, 55, 56). It was shown that class IIa bacteriocins bind a single extracellular loop in the IIC protein of the Man-PTS, whereas lactococcin A binds many regions of IIC and IID proteins for target specificity (57). The Man-PTS found in the inner membrane of sensitive *E. coli* strains, also functions as a target for the microcin E492 (58).

A recent study described a global transcriptional analysis of *L. monocytogenes* isolates which developed spontaneous resistance to the bacteriocin sakacin P (18).

Similar to our findings, Tessema et al. observed diminished growth rates of sakacin P-resistant *L. monocytogenes* isolates on mannose while no difference was found when grown on cellobiose in that study (18). The Man-PTS, apart from acting as a receptor for class IIa bacteriocins such as sakacin P as mentioned above, is also involved in transporting mannose (18). In another study, Kjos and co-workers isolated *L. lactis* and *L. monocytogenes* mutants resistant to lactococcin A and noted that the mechanisms of resistance to lactococcin A was associated with attenuated expression of Man-PTS (16). However, in the case of some lactococcin A-resistant mutants, expression of Man-PTS was not affected and the mechanisms of resistance amongst such mutants remain unclear. Nonetheless, a connection between resistance development and altered metabolism of different sugar substrates was apparent in the study (16). Other studies have also reported that resistance to class IIa bacteriocins in *L. monocytogenes* is associated with downregulation of the Man-PTS genes (17, 23, 59).

The ability of target strains to develop resistance or attenuated sensitivity to bacteriocins is a potential problem in clinical settings and especially in the food industry, where bacteriocins such as nisin are already used. Serial exposure to nisin leading to the development of stable mutants of *Streptococcus pneumoniae* was described in a study (60). While it is clear that alterations in lipid II are not involved in the development of resistance to nisin, more recent studies have highlighted the roles of Nisin-Resistant Proteins and two component systems involved in attenuated sensitivity to the lantibiotic (32, 34, 59). Similar to nisin-resistant *S. pneumonia*e mutants described by Severina and co-workers, thuricin CD-resistant mutants in this study have also been isolated by serially exposing sensitive strains to increased thuricin CD concentrations (41). Alterations in the cell envelope, such as changes in

the thickness of the cell wall, fluidity and charge of the cell membrane and/or cell wall or combination of the aforementioned factors are generally attributed to the development of resistance to bacteriocins (24, 28, 60, 61, 62, 63, 64). A combination of the above may be involved in the development of thuricin CD-resistant mutants described in this study. Cross-resistance to other bacteriocins and/or environmental stresses is also a potential problem, especially in the food industry whereby the use of hurdle technology is of paramount importance in order to preserve food. It is noteworthy in this regard that Lrm1, Lrm2 and Lrm3 did not display increased tolerance to salt or pH stress.

In conclusion, this study gives a detailed account of the development of low-level resistance to the sactibiotic thuricin CD amongst five sensitive strains and also reports the MICs of a range of antimicrobials against these five indicators and their respective thuricin CD-resistant mutants. Thuricin CD-resistant mutants of *L. monocytogenes* 33013, *B. firmus* NRS854, *C. difficile* Liv024 R001, *C. difficile* TL174 R015 and *C. difficile* CD196 R027 displayed two-fold to eight-fold increases in thuricin CD MICs. The emergence of thuricin CD-resistant mutants due to repeated exposure to the bacteriocin, as described in this study, may provide an insight into the mechanism of action of this unusual bacteriocin, while also providing information on how thuricin CD should be deployed in a potential clinical setting.

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Tables and Figures

Table 1: Minimum inhibitory concentration values of antimicrobials against *C. difficile* and thuricin CD-resistant mutants. MICs of several antimicrobials against *C. difficile* Liv024 R001, *C. difficile* TL174 R015, *C. difficile* CD196 R027 and their thuricin CD-resistant mutants. MIC values are indicated in µM with µg/ml values included in parentheses. # Strain name, followed by ribotype. ## Res; Resistant mutant.

C. difficile strain#	Liv024 R001 µM/ **(µg/ml)**	Liv024 R001 Res. µM/ **µg/ml)**	TL174 R015 µM/**(µg/ml)**	TL174 R015 Res.## µM/**(µg/ml)**	CD196 R027 µM/ **(µg/ml)**	CD196 R027 Res. µM/ **(µg/ml)**
Thuricin CD	0.5 **(2.812)**	2 **(11.248)**	0.25 **(1.406)**	1.5 **(8.436)**	0.25 **(1.406)**	0.5 **(2.812)**
Trnα	2.25 **(6.2)**	>4.5 **(>12.4)**	4.5 **(12.4)**	>4.5 **(>12.4)**	2.25 **(6.2)**	4.5 **(12.4)**
Trnβ	4.5 **(12.43)**	>4.5 **(>12.43)**	>4.5 **(>12.43)**	>4.5 **(>12.43)**	4.5 **(12.43)**	4.5 **(12.43)**
CA[a]	3.9 **(1.26)**	3.9 **(1.26)**	7.8 **(2.52)**	7.8 **(2.52)**	3.9 **(1.26)**	3.9 **(1.26)**
Ceftazidime	125 **(68.3)**	125 **(68.3)**	125 **(68.3)**	62.5 **(34.15)**	62.5 **(34.15)**	62.5 **(34.15)**
Oxacillin	25 **(10.58)**	25 **(10.58)**	25 **(10.58)**	25 **(10.58)**	50 **(21.16)**	50 **(21.16)**
Penicllin G	1.95 **(0.696)**	0.975 **(0.348)**	0.975 **(0.348)**	0.975 **(0.348)**	0.975 **(0.348)**	0.975 **(0.348)**
Tetracycline	0.366 **(0.160)**	0.366 **(0.160)**	0.366 **(0.160)**	0.366 **(0.160)**	0.366 **(0.160)**	0.366 **(0.160)**
Erythromycin	250 **(184)**	250 **(184)**	3.9 **(2.86)**	3.9 **(2.86)**	3.9 **(2.86)**	3.9 **(2.86)**
Cephradine	250 **(87.25)**	250 **(87.25)**	125 **(43.625)**	125 **(43.625)**	125 **(43.625)**	125 **(43.625)**
Actagardine	6.25 **(11.813)**	6.25 **(11.813)**	3.125 **(5.907)**	3.125 **(5.907)**	3.125 **(5.907)**	6.25 **(11.813)**
Ramoplanin	0.312 **(0.704)**	0.312 **(0.704)**	0.156 **(0.352)**	0.312 **(0.704)**	0.312 **(0.704)**	0.312 **(0.704)**
Bacitracin	>25 **(>35.55)**	>25 **(>35.55)**	>25 **(>35.55)**	>25 **(>35.55)**	>25 **(>35.55)**	>25 **(>35.55)**
Metronidazole	2.5 **(0.428)**	2.5 **(0.428)**	1.25 **(0.214)**	1.25 **(0.214)**	1.25 **(0.214)**	0.625 **(0.107)**
Vancomycin	0.625 **(0.928)**	0.625 **(0.928)**	0.625 **(0.928)**	0.312 **(0.464)**	0.625 **(0.928)**	1.25 **(1.856)**
Ampicillin	1.95 **(0.723)**	1.95 **(0.723)**	1.95 **(0.723)**	1.95 **(0.723)**	0.975 **(0.362)**	0.975 **(0.362)**
Lysozyme	>2096 **(>3000)**	>2096 **(>3000)**	>2096 **(>3000)**	>2096 **(>3000)**	>2096 **(>3000)**	>2096 **(>3000)**

[a]CA, chloramphenicol

Table 2: Minimum inhibitory concentration values of antimicrobials against *B. firmus* NRS854 and thuricin CD-resistant mutants. MIC figures are indicated in µM with µg/ml values included in parentheses. Mutants designated Bf1-Bf5.

Strain	*B. firmus* Wt µM /**(µg/ml)**	Bf1 µM/**(µg/ml)**	Bf2 µM/**(µg/ml)**	Bf3 µM /**(µg/ml)**	Bf4 µM/**(µg/ml)**	Bf5 µM /**(µg/ml)**
Thuricin CD	0.125 **(0.703)**	0.5 **(2.812)**	0.5 **(2.812)**	0.5 **(2.812)**	1 **(5.624)**	1 **(5.624)**
CA[a]	7.5 **(2.42)**	7.5 **(2.42)**	7.5 **(2.42)**	7.5 **(2.42)**	7.5 **(2.42)**	7.5 **(2.42)**
Ery[b]	25 **(18.35)**	25 **(18.35)**	12.5 **(9.18)**	25 **(18.35)**	25 **(18.35)**	25 **(18.35)**
Pen G[c]	15.5 **(5.518)**	7.75 **(2.76)**	7.75 **(2.76)**	7.75 **(2.76)**	3.875 **(1.38)**	3.875 **(1.38)**
Oxacillin	12.5 **(5.288)**	6.25 **(2.64)**	6.25 **(2.64)**	6.25 **(2.64)**	3.125 **(1.32)**	3.125 **(1.32)**
Ampicillin	12.5 **(4.638)**	6.25 **(2.32)**	6.25 **(2.32)**	6.25 **(2.32)**	3.125 **(1.16)**	3.125 **(1.16)**
Cephradine	0.625 **(0.218)**	0.625 **(0.218)**	1.25 **(0.436)**	0.625 **(0.218)**	0.625 **(0.218)**	0.625 **(0.218)**
Ceftazidime	5 **(2.73)**	1.25 **(0.683)**	5 **(2.73)**	5 **(2.73)**	2.5 **(1.366)**	5 **(2.73)**
CPC[d]	3.49 **(1.25)**	3.49 **(1.25)**	3.49 **(1.25)**	3.49 **(1.25)**	3.49 **(1.25)**	3.49 **(1.25)**
Lysozyme	>2096 **(>3000)**	>2096 **(>3000)**	>2096 **(>3000)**	>2096 **(>3000)**	>2096 **(>3000)**	>2096 **(>3000)**

[a]CA, chloramphenicol; [b]Ery, erythromycin, [c]Pen G, penicllin G; [d]CPC, cetypyridinium chloride

Table 3: Minimum inhibitory concentrations of antimicrobials against *L. monocotogenes* 33013 and thuricin CD-resistant mutants. MIC values are indicated in µM with µg/ml values included in parentheses. Mutants designated Lmr1-Lmr3.

Strain	L.m Wt µM/ **(µg/ml)**	Lmr1 µM/ **(µg/ml)**	Lmr2 µM/ **(µg/ml)**	Lmr3 µM/ **(µg/ml)**
Thuricin CD	2.5 **(14.06)**	5 **(28.12)**	10 **(56.24)**	20 **(112.48)**
Penicillin G	0.78 **(0.278)**	0.78 **(0.278)**	0.78 **(0.278)**	0.78 **(0.278)**
Oxacillin	6.25 **(2.64)**	6.25 **(2.64)**	6.25 **(2.64)**	6.25 **(2.64)**
Ampicillin	0.78 **(0.289)**	0.78 **(0.289)**	0.78 **(0.289)**	0.78 **(0.289)**
Cephradine	125 **(43.675)**	125 **(43.675)**	125 **(43.675)**	62.5 **(21.838)**
Ceftazidime	>250 **(136.645)**	>250 **(136.645)**	>250 **(136.645)**	>250 **(136.645)**
Chloramphenicol	15.625 **(5.047)**	15.625 **(5.047)**	15.625 **(5.047)**	15.625 **(5.047)**
Erythromycin	0.097 **(0.071)**	0.195 **(0.143)**	0.195 **(0.143)**	0.195 **(0.143)**
Bacitracin	25 **(71.1)**	25 **(71.1)**	25 **(71.1)**	25 **(71.1)**
Lysozyme	1048 **(1500)**	1048 **(1500)**	1048 **(1500)**	1048 **(1500)**

Fig 1:

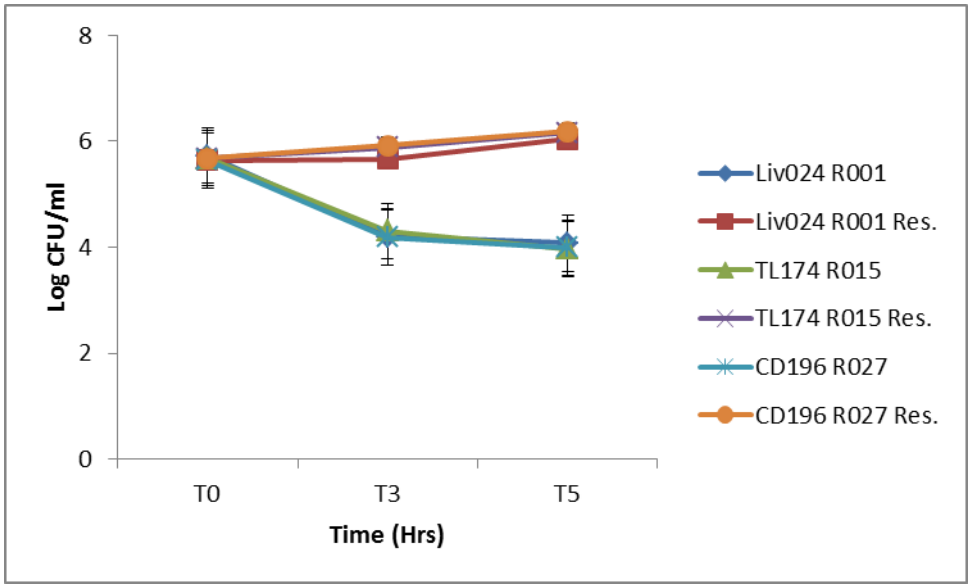

Fig 1: Kill curve analysis of *C. difficile* strains and respective thuricin CD-resistant mutants. Survival and growth of *C. difficile* Liv024 R001, *C. difficile* TL174 R015, *C. difficile* CD196 R027 and their respective thuricin CD-resistant mutants when challenged with 0.225µM (for TL174 R015 and CD196 R027) and 0.45µM (for Liv024 R001) thuricin CD. Res; Thuricin CD-resistant mutants.

Fig 2:

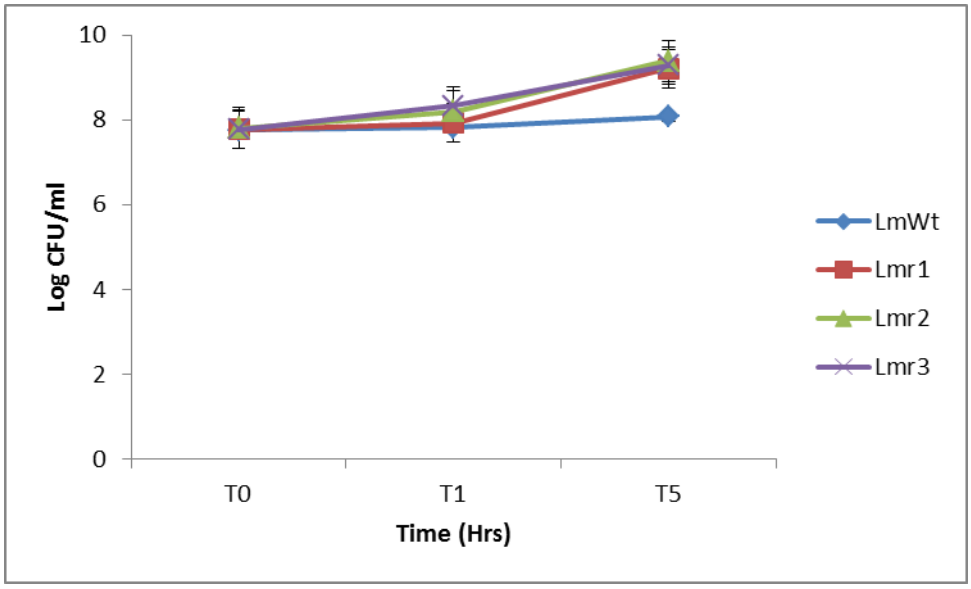

Fig 2. Survival studies of *L. monocytogenes* 33013 and thuricin CD-resistant mutants. Survival and growth of *L. monocytogenes* 33013 and its thuricin CD-resistant mutants when challenged with a sub-lethal dose of thuricin CD (2.4µM). Mutants designated Lmr1-Lmr3.

Fig 3:

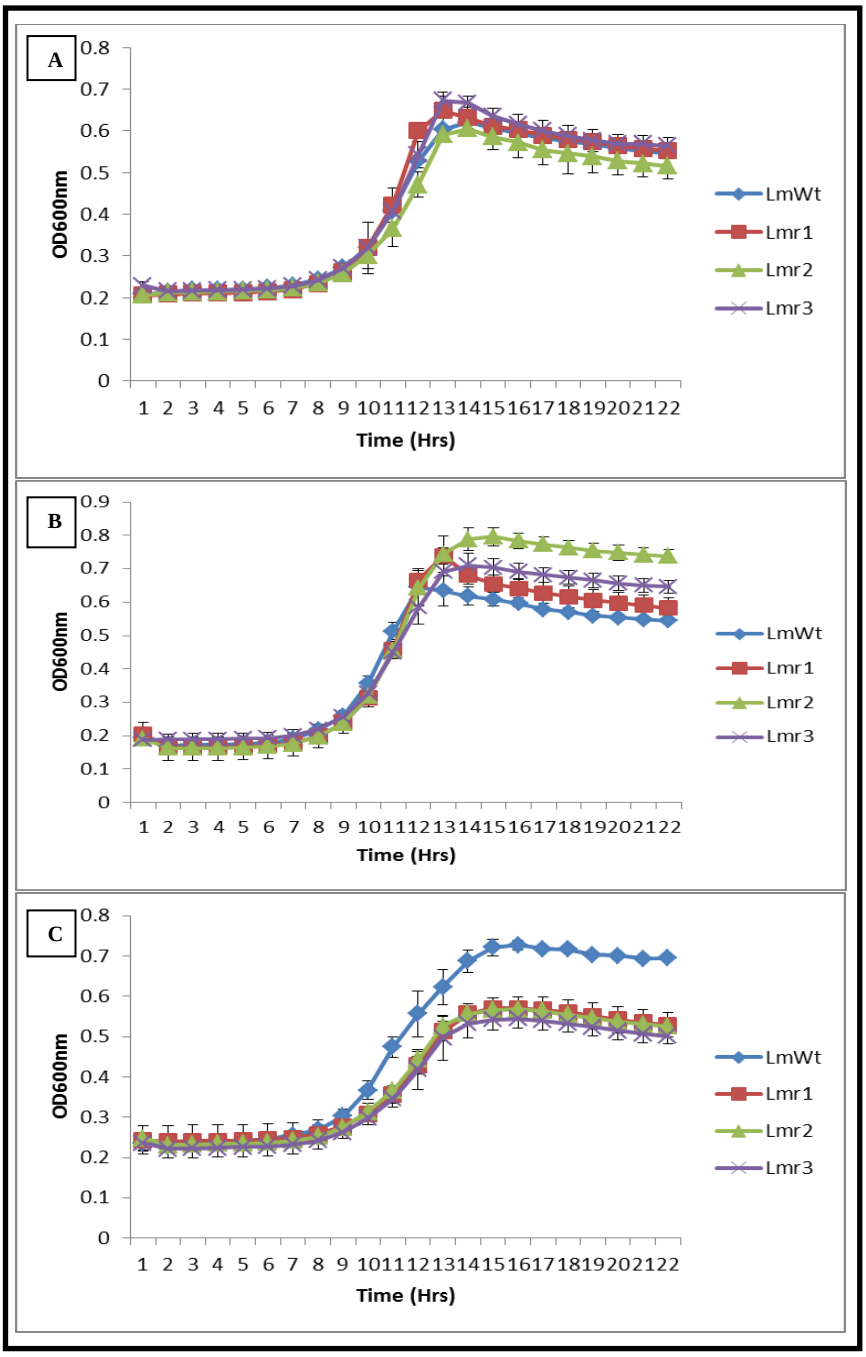

Fig 3:

Growth curve analysis of *L. monocytogenes* 33013 and thuricin CD-resistant mutants. Growth curve of *L. monocytogenes* 33013 and its thuricin CD-resistant mutants when grown in Brain Heart Infusion broth, supplemented with 5% mannose (A), 5% galactose (B) or 5% glucose (C).

Fig 4:

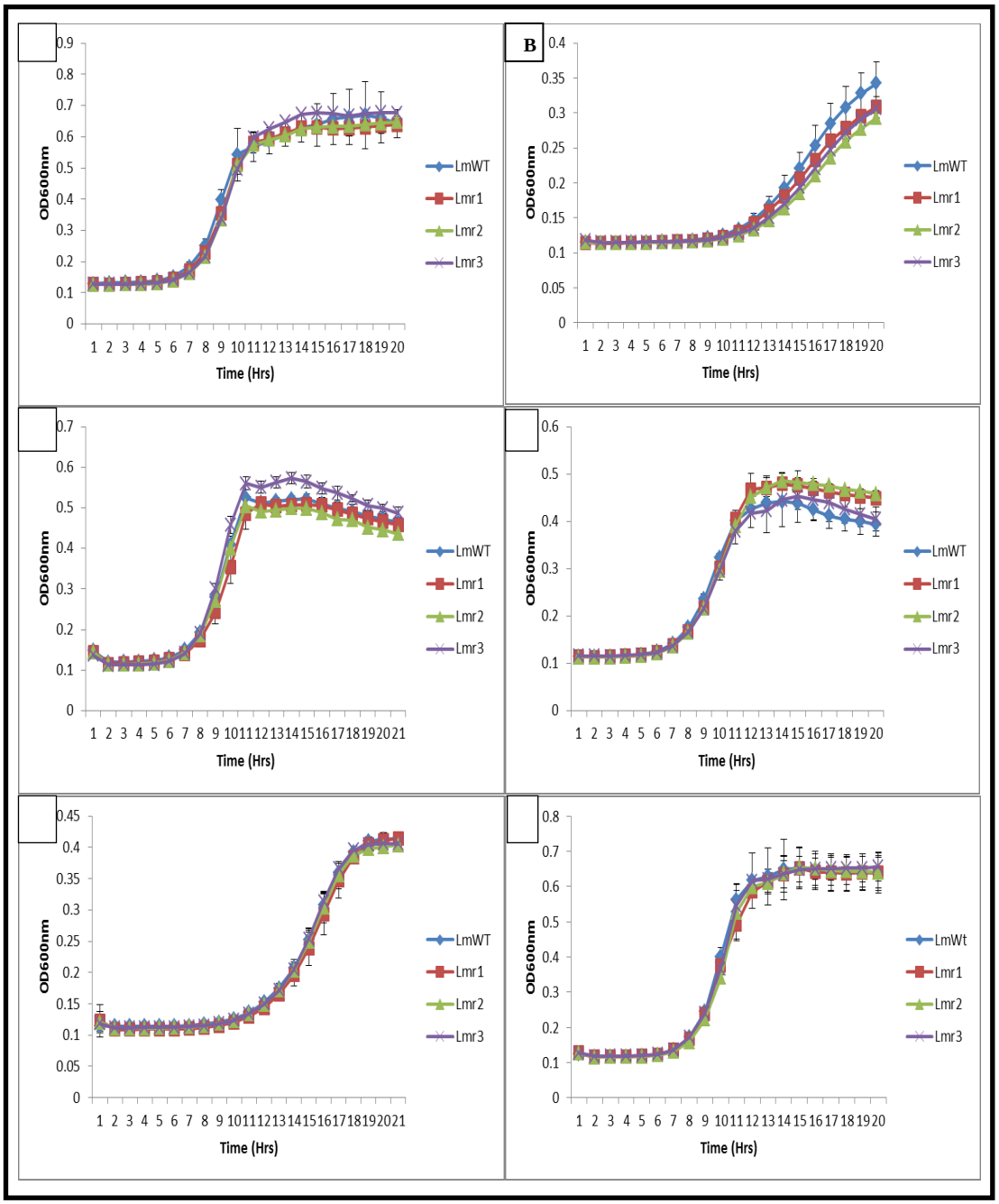

Fig 4: Growth kinetics of *L. monocytogenes* 33013 and thuricin CD-resistant mutants under stressful conditions. Growth curve analysis of *L. monocytogenes* 33013 and its thuricin CD-resistant mutants when grown in Brain Heart Infusion broth at pH5 (A), pH6 (B), pH7 (C), supplemented with 6.25% sodium chloride (D), supplemented with 625 µg/ml lysozyme (E) or supplemented with 0.256 µg/ml cetylpyridinium chloride.

Thesis Summary

The recurrence of CDAD post-antibiotic use is an ongoing problem that must be addressed. CDAD is primarily caused by broad spectrum antibiotics, often leading to perturbations of the commensal gut microbiota, resulting in a cyclic pattern of recurrent CDAD. This thesis describes the work conducted with thuricin CD, a narrow spectrum bacteriocin with potent anti-*C. difficile* activity. As a result of these attributes, thuricin CD has the potential to replace or at least be used in conjunction with traditional antibiotics such as metronidazole and vancomycin for treatment of CDAD. This thesis describes the developments made with respect to thuricin CD, including the heterologous expression of thuricin CD, the identification of the thuricin CD immunity genes, insights into the mechanisms of development of low-level resistance to thuricin CD and its effectiveness in combating *C. difficile*, when used in combination with other antimicrobials. In addition, the thesis contributes to our understanding of the biology of the newly-designated sactibiotic group of bacteriocins.

Chapter I describes the processes involved in *C. difficile* infection, focussing on hypervirulent and toxin-variant strains, toxin production, diagnosis, traditional treatment modalities and novel/alternative treatment options which may be used in the future for CDAD. In particular, it focuses on the mechanisms of pathogenesis of hypervirulent/toxin-variant *C. difficile* strains and describes the potential of novel antimicrobials such as fidaxomicin, ramoplanin, tigecycline as well as bacteriocins such as thuricin CD, LFF571 and NVB302. Furthermore, the advantages/disadvantages of novel treatment options are discussed. In addition, the potential of alternative adjunctive treatment options such as probiotics, faecal bacteriotherapy, ion-binding resins and vaccines are discussed in Chapter I.

Chapter II focuses on antimicrobial combination studies against a range of *C. difficile* clinical isolates. In this chapter, the MICs of thuricin CD against 19 *C. difficile* isolates are determined and compared with the three antibiotics metronidazole, vancomycin and ramoplanin as well as against the bacteriocin actagardine, belonging to the lantibiotic group. These MICs indicate that in terms of molar concentrations, thuricin CD consistently performs better than metronidazole, vancomycin and actagardine. Ramoplanin yields the lowest MICs against the majority of the *C. difficile* isolates tested in this study in terms of molar concentrations. Antimicrobial combination studies using the five antimicrobials mentioned above were conducted against 13 *C. difficile* clinical isolates using broth-based checkerboard assays, resulting in a total of 117 combinations assessed. Out of these 117 combinations, 34 were found to have partial synergistic/additive effects. Significantly, ramoplanin/actagardine combinations proved particularly effective with partial synergistic/additive effects obtained against 61.5% of *C. difficile* strains tested. Although it has been acknowledged that there are limitations with *in vitro* studies, especially with *C. difficile*, the findings of this study could form the basis for downstream *in vivo* studies with murine/porcine models of infection and possibly clinical trials, with the ultimate goal to find effective combinations to be used in the clinic against cases of CDAD.

Chapter III focused on gaining a better insight into the immunity systems employed by *B. thuringiensis* DPC6431, to protect itself from the antimicrobial actions of thuricin CD i.e. to prevent self-killing. A number of constructs encompassing the putative thuricin CD immunity determinants were constructed and introduced into the thuricin CD-sensitive indicator *L. monocytogenes* 33013. The results from this study helped to identify the immunity genes in the thuricin CD gene cluster. It

emerged that four genes are involved in providing protection against thuricin CD. Significantly, as part of this study, it emerged that an open reading frame located upstream of *trnF* is in fact an immunity gene, encoding a small hydrophobic transmembrane immunity protein. Extensive *in silico* analysis of this novel protein, designated TrnI, indicated that it consists of two transmembrane helices and is predicted to function by aggregating thuricin CD or somehow interfering with the ability of the thuricin CD peptides to bind the target site/receptor. This dedicated immunity protein TrnI may function in a similar manner to LanI proteins in lantibiotic immunity systems. Further studies will help to disclose the precise mechanisms by which the newly-discovered TrnI peptide functions in providing immunity. *In silico* analysis of TrnG also indicated that it is a hydrophobic protein, consisting of 6 transmembrane helices and forms the integral membrane domain of a fully functional ABC-transporter system TrnFG, also involved in thuricin CD immunity.

The main focus of Chapter IV was the cloning and expression of thuricin CD in a heterologous host. Since the thuricin CD producer is *B. thuringiensis* DPC6431, it appeared logical to employ a *Bacillus* strain as a heterologous host. Due to its insensitivity to thuricin CD, compounded by its ability to express heterologous proteins and its GRAS status, *B. subtilis* 1012 was chosen as a heterologous host and the IPTG-inducible pHCMC05 expression vector was selected to this end. Cloning of the full length of the thuricin CD gene cluster, encompassing the putative biosynthetic, immunity, post-translational modification and export machinery into pHCMC05 resulted in the production of thuricin CD in the *B. subtilis* 1012 host. This result confirmed that the eight genes of the putative thuricin CD gene cluster were essential for the production of biologically active thuricin CD. Downstream

assays were conducted to compare the levels of production of thuricin CD by the natural producer and the heterologous host, and it emerged that the *B. subtilis* host failed to produce thuricin CD at alkaline pH conditions. Cloning of truncated versions of the full construct, with either the ABC-transporter genes or the post-translational modification genes excluded, resulted in loss of thuricin CD production in the *B. subtilis* host. This 'proof of concept'-type study confirmed that each of the genes in the thuricin CD gene cluster are essential for production. Furthermore, this development of a system for the heterologous production of thuricin CD will allow future random/site-directed mutagenesis studies to be conducted, in order to isolate variants with enhanced bioactivity.

As part of Chapter V of this thesis, low-level thuricin CD resistant mutants across three sensitive species were isolated, with the ultimate view to gain an insight into the mode of action of thuricin CD. Low-level thuricin CD resistant mutants of *L. monocytogenes* 33013, *B. firmus* NRS854, *C. difficile* Liv024 R001, *C. difficile* TL174 R015, *C. difficile* CD196 R027 were isolated by exposing the strains to incrementally increasing concentrations of thuricin CD in a stepwise manner. It was apparent from phenotypic assays that minor variations in sensitivities to different antimicrobials did exist amongst the thuricin CD-resistant mutants. However, a lack of a direct link between attenuated sensitivity to thuricin CD and slightly altered sensitivity to other antimicrobials suggests that different mechanisms of resistance development may be involved amongst different mutants. Nonetheless, altered growth rates of thuricin CD-resistant *L. monocytogenes* mutants in the presence of the sugars mannose and glucose suggests that the mannose phosphotransferase system may be a potential receptor for thuricin CD.

Overall, the work presented in this thesis answers some of the fundamental questions about thuricin CD biology, genetics, development of attenuated sensitivity amongst target strains and efficacy when used in combination with other antimicrobials. Such findings could have significant implications in the potential use of thuricin CD in the clinic. The major issues relating to the treatment failure of CDAD have been highlighted and this thesis addresses these issues by seeking alternative therapeutic combinations and investigating the propensity for thuricin CD resistance development. Furthermore, the development of a heterologous host system and the identification of the immunity systems employed by the thuricin CD producer may serve as a foundation for the eventual over-production of thuricin CD. Such developments will help to expedite the processes involved in the deployment of thuricin CD in the clinic.

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Harsh.